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BIOCHEMICAL CHANGES IN INBORN AND ACQUIRED ERRORS OF METABOLISM

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ACADEMIC DISSERTATION

To be presented, with the permission of the Medical Faculty of the University of Helsinki, for public examination in the Seth Wichmann auditorium at the Department of Obstetrics and Gynaecology, Helsinki University Hospital, on May 4th 2012, at 12 noon.

Helsinki 2012

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ISBN (Paperback) 978-952-10-7913-9
ISBN (PDF) 978-952-10-7914-6
http://ethesis.helsinki.fi
Unigrafia Oy
Helsinki 2012

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1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by their Roman numeral.

I
Salmi H, Hussain K, Lapatto R. Changes in plasma and erythrocyte thiol levels in children undergoing fasting studies for investigation of hypoglycaemia. Pediatr Endocrinol Diabetes Metab 2011;17(1):14-9.

II
Salmi H, Leonard JV, Rahman S, Lapatto R. Plasma thiol status is altered in children with mitochondrial diseases. Scand J Clin Lab Invest 2012 Jan 2 (Epub ahead of print).

III
Salmi H, Leonard JV, Lapatto R. Patients with organic acidaemias have an altered thiol status. (submitted)

IV
Salmi H, Kuitunen M, Viljanen M, Lapatto R. Cow's milk allergy is associated with changes in urinary organic acid concentrations. Pediatr Allergy Immunol. 2010 Mar;21(2 Pt 2):e401-6. Epub 2009 Apr 22.

In addition, some previously unpublished results are presented.

2. LIST OF NONSTANDARD ABBREVIATIONS

CBS	cystathionine β-synthase (EC 4.2.1.22)
CM	cow's milk
CMA	cow's milk allergy
CYSH	reduced cysteine
CYSS	(oxidised) cystine (disulphide)
DBPCFC	double-blind placebo-controlled food challenge
ETC	electron transport chain
G6PDH	glucose 6-phosphate dehydrogenase
GCS	γ-glutamylcysteine synthetase, also called glutamate-cysteine ligase (EC 6.3.2.2)
GPx	glutathione peroxidase(s) (EC 1.11.1.9)
GR	glutathione reductase (EC 1.8.1.7)
GS	glutathione synthetase (EC 6.3.2.3)
GSH	reduced glutathione
GSSG	glutathione disulphide
GST	glutathione transferase(s) (EC 2.5.1.18)
HPLC	high-pressure liquid chromatography
IVA	isovaleric acid/ -aemia
LGG	*Lactobacillus rhamnosus GG*
MDA	malondialdehyde
MMA	methylmalonic acid/ -aemia
mtDNA	mitochondrial DNA
nDNA	nuclear DNA
PA	propionic acid/ -aemia
SCFA	short-chain fatty acid
SOD	superoxide dismutase (EC 1.15.1.1)
SPT	skin prick test
TBA-RS	thiobarbituric acid –reactive species
γGT	γ-glutamyl transpeptidase, also called γ-glutamyltransferase (EC 2.3.2.2)

3. ABSTRACT

Normal human metabolism is a well-integrated process involving the whole organism. It can be disturbed in very different situations, either in true inborn errors of metabolism or in acquired situations where abnormal cells or tissues produce abnormal metabolites, or during critical illness, when overwhelming metabolic stress is present in normal tissues. These situations may share common pathogenetic mechanisms and metabolic markers, and their closer knowledge could provide new approaches for treatment or diagnosis.

Objectives

This thesis had two objectives, both dealing with biochemical changes in situations where significant metabolic perturbations occur.

(1) To study thiol metabolism in children with several inborn errors of metabolism (organic acidaemias, hypoglycaemic episodes, mitochondrial diseases, homocystinuria) and in cell culture models, where similar metabolic conditions are created. Thiol levels and thiol redox status, serving multiple metabolic and regulatory purposes, could be affected in inborn errors of metabolism following inappropriate nutrition and compromised energy metabolism. Possible changes in thiol status and the associated increase in oxidative stress could have an important role in the development of complications in these diseases. Changes in thiol status could even offer therapeutic potential, as thiol levels can be influenced by diet or medication.

(2) To investigate end products of metabolism in infants with cow's milk allergy (CMA) as metabolic markers of inadequate nutrition in early childhood and altered intestinal microbial metabolism. Eventually, these changes could provide a novel diagnostic tool.

Methods

Children (n = 36) with inborn errors of metabolism (either organic acidaemia, mitochondrial disease, homocystinuria or diagnosed hypoglycaemia) were enrolled from Great Ormond Street Hospital, London, UK. Infants (n = 35) with diagnosed or

suspected cow's milk allergy participated from Skin and Allergy Hospital, Helsinki, Finland. Patients referred to the same clinic but in whom no disease was observed formed the respective control groups.

Plasma and erythrocyte non-protein thiols (glutathione and cysteine) as well as thiols in cultured human fibroblasts, HEPG2 and 293T cells were measured with a liquid chromatography –based method. Mass spectrometry was used for quantification of urinary excretion of end products of metabolism in CMA patients. Activities of enzymes related to thiol metabolism in human erythrocytes were studied in spectrophotometric assays.

Results

(1) Patients with organic acidaemias, hypoglycaemic episodes, mitochondrial diseases and homocystinuria had altered levels of plasma non-protein thiols glutathione and cysteine, and their plasma thiol redox status was indicative of oxidative stress even in the absence of acute critical illness.

(2) Patients with CMA had increased urinary excretion of several metabolic end-products. These changes in urinary metabolic profile may reflect inappropriate nutrition, altered intestinal bacterial metabolism or increased intestinal permeability.

Conclusions

(1) Thiol metabolism is altered in several inborn errors of energy and nutrient metabolism. The changes in thiol redox status are suggestive of oxidative stress, which seems to play a role in the pathogenesis of these diseases or their complications.

(2) With further research, the changes in thiol levels and thiol redox state could have therapeutic implications; thiol status can be affected by dietary sulphur amino acid intake and thiol antioxidant supplementation.

(3) CMA is associated with measurable changes in urinary levels of end products of metabolism, which may be seen as markers of inadequate nutrition. With further research, these changes could provide an innovative new approach to the diagnosis of CMA

4. INTRODUCTION

Metabolism is a delicately coordinated entity of chemical reactions. By these reactions, the organism produces the necessary elements for tissue maintenance, growth and reproduction, excretes toxic and residual compounds and maintains a balance between energy and nutrient intake, consumption and storage. Human metabolism can be viewed and studied either from the perspective of the whole organism, certain tissues or individual cells or cell populations. In an experimental setting, cultured cells give information about metabolism in individual cells or cell populations. Evaluation of plasma values is more suggestive of the overall metabolic situation at a given time, whereas quantification of end products excreted in urine gives an overview of long-time metabolic homeostasis in the whole organism (Maher et al. 2007).

Metabolism can be disturbed in either inherited or acquired situations. At present, the majority of inherited metabolic diseases cannot be cured, and many of them have a fatal course. When available, the treatment often remains supportive and experimental. These diseases cause severe disturbances in growth and development and often present with episodes of acute metabolic decompensation. The detailed pathogenesis of these severe, even fatal, complications is often unclear, making possibilities for treatment few. New information about the pathogenesis of the complications in these diseases could be valuable, as it could provide new therapeutic approaches.

Research on metabolic diseases may also be useful for a thorough understanding of normal human metabolism. Many metabolic disturbances in originally "non-metabolic" diseases may have common pathogenetic factors with inborn errors of metabolism. Metabolism and nutrition are also closely related, as situations with disturbed metabolism are often associated to inappropriate nutrition, and poor nutrition leads to metabolic changes. Studying metabolic disturbances may, then, also reveal the underlying nutritional problem.

This thesis has focused on biochemical changes in different situations with significant metabolic disturbances. These changes are seen as metabolic markers of the underlying pathogenetic processes, offering new insights into disease mechanisms and, with further research, even therapeutic potential.

5. REVIEW OF THE LITERATURE

5.1. INBORN ERRORS OF METABOLISM

Inborn errors of metabolism are rare congenital diseases that are mainly due to a genetic defect of enzymes or cofactors participating in a certain metabolic pathway or the transport of metabolites within a cell or between cells. This can lead to

1. **Accumulation of substrate** (e.g. accumulation of methylmalonate in methylmalonyl-CoA mutase deficiency, methylmalonic acidaemia)
2. **Loss of end product** (e.g. hypoglycaemia following defects in gluconeogenesis, or defective oxidative ATP energy production in mitochondrial diseases)
3. **Accumulation of normally minor metabolites** (e.g. accumulation of propionic acid in methylmalonic acidaemia)
4. **Secondary metabolic consequences** (acidosis, ketosis, accumulation of lactate or ammonia). These may obscure the underlying metabolic disturbance and cause diagnostic confusion.

The ensuing symptoms often appear during the neonatal period but sometimes only later, often in the childhood, giving rise to late-onset forms. Also adult-onset inherited metabolic diseases exist; in these diseases, the symptoms are often chronic and progressive. Most inborn errors of metabolism, however, affect infants and young children.

One of the major problems in understanding inherited metabolic disorders is their enormous variability. Individual inherited metabolic diseases are very rare but, collectively, they form an important group of diseases especially amongst paediatric diseases. Many different types of inherited metabolic diseases can give rise to similar symptoms and cause diagnostic confusion; clinical phenotypes of single diseases may also vary. Rather than classifying metabolic disorders according to their pathogenesis or to the most striking but often varying clinical features they have (Clarke 2006), a useful approach is to divide metabolic disorders according to their main presentation in clinically oriented pathogenetic subgroups (Saudubray et al. 2006). This approach is also suitable for guiding therapeutic measures.

1. **Disorders of "intoxication type"**, where the accumulation of a substance that is toxic when present in excess is the main clinical problem (e.g. urea cycle disorders, aminoacidopathies such as phenylketonuria, organic acidaemias, metal disorders, sugar intolerances). An initial symptom-free period is

followed by intoxication-like symptoms, which may be acute or chronic, but often intermittent and provoked by acute illness or dietary changes. The treatment, if available, relies on nutritional therapy. A special diet may reduce the accumulation of the toxic substance (e.g. avoiding phenylalanine and its precursors in phenylketonuria). Sometimes, cofactor supplementation may boost residual enzymatic activity to allow sufficient enzymatic function (e.g. the B_{12} –responsive form of methylmalonic acidaemia). It may also be possible to enhance the excretion of the accumulating metabolite by nutritional supplements or medication (e.g. the use of sodium benzoate in hyperammonaemia), or to replace a critical intermediary metabolite and thus reduce the accumulation of other metabolites in a specific metabolic pathway (e.g. supplementation of citrulline in some urea cycle disorders).

2. **Disorders of energy metabolism**, either mitochondrial defects (disorders of the respiratory chain, Krebs cycle and pyruvate oxidation as well as defects in fatty acid oxidation and ketone bodies) or diseases affecting cytoplasmic energetic processes (e.g. gluconeogenesis and glycolysis defects, hyperinsulinism). In these disorders, the possibilities for treatment depend on the nature of the defect in energy production; most mitochondrial defects are untreatable, whereas defects of gluconeogenesis and glycolysis are often less severe and amenable to treatment.
3. **Disorders involving intracellular organelles and storage,** interfering with synthesis or breakdown of complex molecules. Examples include lysosomal storage diseases, peroxisome diseases and inborn errors of cholesterol synthesis. These disorders have a chronic and progressive course, and are unrelated to diet or other environmental factors. Previously, these disorders were also untreatable, but currently, enzyme replacement therapy is available for some lysosomal storage diseases (Fabry, Gaucher and Pompe diseases and mucopolysaccaridoses I, II and VI; reviewed by Lachman in 2011).

In this series of studies, patients with three distinctive groups of metabolic disturbance have been studied:

(I) organic acidaemias
(II) mitochondrial diseases
(III) hypoglycaemia

In addition, patients with **homocystinuria** were studied, and their previously unpublished results are reported.

5.1.1. ORGANIC ACIDAEMIAS

Organic acidaemias, also called organic acidurias, are rare inborn errors affecting mainly the catabolism of branched chain amino acids (valine, leucine and isoleucine); hence the term branched-chain organic acidurias, which include as well the "classical organic acidurias" (isovaleric, methylmalonic and propionic acidurias) as maple syrup urine disease (MSUD), 3-methylglutaconic acidurias, 3-hydroxy-3-methylglutayl-CoA lyase deficiency, 3-methylcrotonylglycinuria and some rarer conditions. The incidence of all individual organic acidaemias separately or the overall incidence of organic acidaemias are not exactly known, but any organic acidemia is estimated to occur in about 1: 40 000 live births (Zytkovicz et al. 2001). When risk populations such as children with symptoms suggestive of metabolic disease are selectively screened, organic acidemias seem to be among the relatively common metabolic diseases with a prevalence as high as 3% (Wajner et al. 2009). Individual organic acidaemias are rare; the combined incidence of the three classical organic acidaemias (propionic, methylmalonic and isovaleric) has been reported to be less than 2: 100 000 births in the general population with newborn screening (Dionisi-Vici et al. 2006). As these disorders are genetic and inherited in a recessive autosomal manner, their incidence varies greatly between different populations and can reach even 1:2000 or 1:5000 for propionic acidaemia (Al Essa et al. 1998).

5.1.1.1. Metabolic basis

Due to metabolic blockage following the specific enzyme deficiency in the catabolism of isoleucine, leucine or valine, short chain carboxylic acids and their metabolites accumulate in tissues and are excreted in excess in urine (**Figure 1**). Methylmalonic and propionic acidaemias are not only due to defects in the catabolism of branched-chain amino acids, as propionic acid is also derived from the metabolism of proteins, nucleic acids and lipids and produced by intestinal bacteria, and methylmalonic acid is derived from propionic acid (**Figure 1**) (Sweetman and Williams 2001, Fenton et al. 2001, Ogier de Baulny and Saudubray 2002, Dionisi-Vici et al. 2006). Studies with stable isotopes have confirmed that about 50% of the accumulating propionate and methylmalonate are derived from amino acid catabolism; bacterial metabolism in the gut and catabolism of odd-chain fatty acids in lipids account for the rest in equal amounts (Leonard 1997).

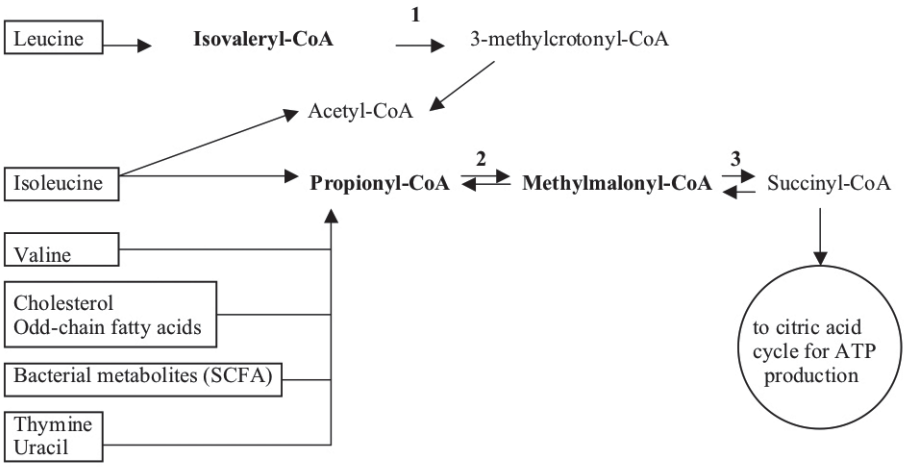


Figure 1. Origins of isovaleric acid (IVA), methylmalonic acid (MMA) and propionic acid (PA) and most important enzymes involved in the development of isovaleric, methylmalonic and propionic acidaemias.

Organic acids (IVA, MMA and/ or PA) are derived from the catabolism of branched-chain amino acids, fats, bacterial metabolites and nucleic acids. Following metabolic blockage in the enzymatic breakdown of IVA, MMA or PA, the corresponding organic acid and its acyl CoA derivative accumulate.
All intermediates are not illustrated.
1 Isovaleryl-CoA dehydrogenase (EC 1.3.99.10)
2 Propionyl-CoA carboxylase (EC 6.4.1.3)
3 Methylmalonyl-CoA mutase (EC 5.4.99.2). B_{12} –derived adenosylcobalamine needed as cofactor.

5.1.1.2. Clinical presentation

Organic acidaemias have an unpredictable course ranging from a severe and often fatal neonatal onset form to milder chronic forms with neurological problems, developmental delay and failure to thrive (Sweetman and Williams 2001; Fenton et al. 2001). The central nervous system is especially affected, with presentations ranging from lethargy to seizures and cerebral palsy or ataxia, and patients often present structural brain abnormalities (Wajner 2004). Also other organ systems may be affected, and patients may have hepatopathy, cardiomyopathy and signs of bone marrow dysfunction. Organic acidaemias often have a fatal course; the mortality rate in patients with either isovaleric, methylmalonic or propionic acidaemias was 51% in a 20-year follow-up study (Dionisi-Vici et al. 2006).

Although the genetic basis and the origin of accumulating metabolites in organic acidaemias are known, the pathogenesis of many complications, including neurological deficits and episodes of acute metabolic crisis, remains unclear. The unexplained and unexpected nature of these complications and their poor correlation to known metabolic parameters, such as the blood concentration of organic acids, makes the clinical management of organic acidaemias very challenging. Some of the pathogenetic mechanisms of specific organic acids will be discussed in the following sections, and the role of oxidative stress in these diseases is reviewed in 5.3 and 5.5.

5.1.1.3. Diagnosis

The diagnosis of organic acidaemias relies on identification of the accumulating organic acids, either in plasma, urine or cerebrospinal fluid, mainly by gas-liquid chromatography and mass spectrometry (GLC-MS). For diagnostic confirmation, determination of the specific enzyme activity in cultured fibroblasts or peripheral leucocytes is useful. Genetic diagnosis is available for known mutations in some of the conditions. Prenatal diagnosis is possible by GLC-MS determination of organic acids in the amniotic fluid, if the genetic defect is not known (Ogier de Baulny and Saudubray 2002). However, even in early-onset severe forms, the symptoms of organic acidaemias are unspecific and easily misinterpreted for infection or other acute illness, delaying the diagnosis. Newborn screening for organic acidaemias, performed by tandem mass spectrometry, may be used, but the long-term effects of newborn screening to the prognosis of organic acidaemias are unknown (Dionisi-Vici et al. 2006).

5.1.1.4. Treatment

The treatment of organic acidaemias consists of minimising the accumulation of the abnormal metabolites by dietary restriction of branched-chain amino acids, and the acute treatment of complications and periods of metabolic decompensation. In addition, specific treatment is available for cobalamin (vitamin B_{12}) -responsive forms of methylmalonic acidaemia, and carnitine may be useful for facilitating the excretion of propionic acid metabolites and to prevent carnitine deficiency following conjugation reactions to carnitine. In propionic and methylmalonic acidaemias, intermittent antibiotic treatment by metronidazole in order to suppress propionate production by the intestinal flora, which accounts for 25% of propionate production (Leonard 1997) seems to be helpful (Ogier de Baulny 2002). As catabolism of odd-chain fatty acids in lipids also results in propionic acid formation, prolonged fasting and other states promoting lipolysis should be avoided and treated promptly in propionic and methylmalonic acidaemias (Leonard 1997).

5.1.1.5. Isovaleric acidaemia (OMIM 243500)

Isovaleric acidaemia (IVA) is caused by a deficiency in the function of the apoenzyme of the mitochondrial enzyme isovaleryl-CoA dehydrogenase (EC 1.3.99.10). The defect leads to accumulation of isovaleric acid, 3-hydroxyisovaleric acid and N-isovalerylglycine and, following conjugation to carnitine, also isovaleryl carnitine. Free IVA is toxic, but formation of N-isovalerylglycine and isovaleryl carnitine leads

to formation of less harmful and soluble metabolites (Ogier de Baulny and Saudubray 2002). Although the mechanism of IVA toxicity is not completely understood, it seems to be related to an impairment of mitochondrial energy metabolism and maintenance of membrane potential; in experimental animals, IVA administration inhibited citric acid cycle and disrupted neuronal Na+K+ATPase function (Ribeiro et al. 2007, Ribeiro et al. 2009).

Patients with isovaleric acidaemia present either with an acute neonatal onset form with uncontrollable metabolic acidosis, or with a late-onset chronic and intermittent form with recurrent episodes of ketoacidosis (Sweetman 2001).

5.1.1.6. Methylmalonic acidaemia (OMIM 25100, 251100, 251110, 277410)

Methylmalonic acidaemia (MMA) is genetically more heterogeneous than other organic acidaemias. It is caused by defects in either methylmalonate metabolism (OMIM 25100), with decreased or absent methylmalonyl-CoA mutase (EC 5.4.99.2) function, or cobalamin (vitamin B_{12}) metabolism. Cobalamin is needed for the synthesis of adenosylcobalamin, which is a cofactor for methylmalonyl-CoA mutase, a mitochondrial enzyme needed for methylmalonate breakdown. As expected from its heterogeneous origins, the clinical picture of MMA is extremely variable, ranging from an asymptomatic condition to a severe neonatal onset disease with metabolic acidosis. Patients with MMA due to defects of the translocation and intracellular synthesis of the active forms of cobalamin often also have elevated homocysteine levels (Fenton et al. 2001), as cobalamin –derived methylcobalamin is needed as a cofactor for methionine synthase which normally regenerates methionine form homocysteine. Patients with cobalamin –dependent forms of MMA are often also cobalamin-responsive and have clinical improvement and decreased MMA levels with B12 therapy (Fowler et al. 2008).

Patients with methylmalonic acidaemia often have several metabolic derangements (e.g. hyperammonaemia, hypoglycaemia, acidosis and hyperglycinaemia) that cannot directly be explained by the metabolic block following methylmalonyl-CoA mutase deficiency. The acidosis is not only due to the accumulation of MMA, which is a weak acid, and levels no higher than 3 mmol/l in plasma have been reported. Instead, it appears that the inhibitory effects of MMA to gluconeogenesis and mitochondrial energy metabolism lead to the development of ketosis, which further aggravates the acidosis (Oberholzer et al. 1967, Fenton et al. 2001).

Accordingly to the poorly understood metabolic abnormalities, the pathogenesis of complications and especially the neurological sequelae in methylmalonic acidaemia are somewhat obscure. Also the exact mechanism of toxicity of MMA is incompletely understood, although it seems to be related to defects in mitochondrial

function. Methylmalonate gives intracellularly rise to malonate, which interferes with mitochondrial energy metabolism by inhibiting succinate dehydrogenase (complex II of the respiratory chain) (McLaughlin et al. 1998). In addition, methylmalonate has direct toxic effects to mitochondrial energy production by inhibiting dicarboxylate carrier and, thus, mitochondrial succinate uptake (Mirandola et al. 2008).

5.1.1.7. Propionic acidaemia (OMIM 606054)

Propionic acidaemia (PA) is due to defects in the propionyl coenzyme A carboxylase (EC 6.4.1.3) gene, either in its α- or β-subunit. This mitochondrial enzyme requires biotin as a cofactor, and also inherited errors of biotin metabolism can lead to defects in propionyl CoA carboxylase function and accumulation of propionic acid (Fenton et al. 2001).

Patients have protein intolerance and suffer from periodic metabolic crises with lethargy, vomiting and ketosis, and they often have developmental delay and other neurological sequelae including seizures. Patients also suffer from recurrent complicated infections suggestive of immune deficiency, and they may have leuko- and thrombocytopenia suggestive of bone marrow suppression.

Some of the accumulating propionic acid may be used for fatty acid synthesis, resulting in abnormal, odd-number fatty acids. During a catabolic state and active lipolysis, they may contribute significantly to the patient's propionic acid load (Ogier de Baulny and Saudubray 2002). Also propionate produced by intestinal microbiota influences propionate levels in patients. In addition to an excess of propionic acid, patients also have hyperglycinaemia and –uria, and additional metabolic features such as hyperammonaemia are often present. The metabolic pathways underlying these features are incompletely understood, as are the exact mechanisms of propionic acid toxicity. Some toxic effects of propionate to mitochondrial function have been reported (Stumpf et al. 1980), so it is reasonable to assume that similar mechanisms than those shown in MMA (described previously) could also underlie the toxicity of PA.

5.1.2. HYPOGLYCAEMIA

Rather than a specific metabolic disease, hypoglycaemia is a serious metabolic disturbance accompanying many other metabolic and originally non-metabolic diseases. Premature neonates and neonates who are either small or large for gestational age are at risk for hypoglycaemia; in addition, hypoglycaemia occurs as an iatrogenic factor in diabetic patients on insulin treatment. It is also a complication and symptom in a number of inborn errors of metabolism such as disorders of

fatty acid oxidation, gluconeogenesis defects, glycogen synthetase deficiency, and hyperinsulinaemia (Saudubray et al. 2002, Sperling 2004, Saudubray et al. 2006). In addition, hypoglycaemia may occur in previously healthy individuals during infections or prolonged fasting and/ or malnutrition. Indeed, all children are prone to develop low blood glucose if fasted for a sufficiently long period; hypoglycaemic children may only represent the low end of the physiologic range of tolerance to fast (Sperling 2004).

5.1.2.1. Definition of hypoglycaemia

The definition of hypoglycaemia is arguable and age-dependent (Cornblath 1990); for instance, neonates often have lower blood glucose than children or adults, and blood glucose concentrations as low as 1.7 mmol/l (30 mg/dl) may be considered normal during the first hours of adaptation to postnatal life (Committee on Fetus and Newborn and Adamkin 2011). Whole-blood glucose levels below **2.7 mmol/l (50 mg/dl)** are often considered hypoglycaemic (Sperling 2004); this corresponds to 10-15% higher plasma glucose levels. No clear-cut limits exist; neurological symptoms due to low blood glucose (neuroglycopaenic symptoms) occur at plasma glucose below **3.0 mmol/l (54 mg/dl)** (Cryer 2007). It is particularly difficult to establish a cut-off value for normal blood glucose in children, as low glucose concentrations are sometimes seen in healthy asymptomatic children following prolonged fasting (Cryer 2009).

5.1.2.2. Effects of hypoglycaemia to the central nervous system

The central nervous system is especially vulnerable to prolonged or recurrent hypoglycaemic episodes such as those encountered in metabolic diseases. The pathogenesis of their complications is not thoroughly understood, but it seems evident that these are caused by more complex mechanisms than simply a failure of intracellular energy production. Presumably, hypoglycaemia leads to a sequence of adverse events causing cellular damage and, eventually, cell death; these events involve glutamate receptor activation and exitotoxicity, free radical production, mitochondrial permeability transmission and other cellular sress responses (Auer 2002, Suh et al. 2007).

Previous *in vitro* or experimental studies on hypoglycaemia have been performed in very low glucose concentrations that rarely occur in clinical situations. For instance, cells of different origin have been grown in a medium containing no glucose (Coleman et al. 2007, Suh et al. 2007), or experimental animals allowed to reach a profound hypoglycaemic coma with isoelectric EEG (Suh et al. 2007, Suh et al.

2008). In addition, in experimental settings, *in vivo* hypoglycaemia is often achieved by insulin administration; insulin prevents lipolysis and ketogenesis (Sperling 2004) and deprives the central nervous system from its alternative fuel, ketone bodies. Thus, the effects of insulin-induced (or hyperinsulinaemic) hypoglycaemia to the central nervous system may differ from hypoglycaemia with low insulin levels.

Clinical studies with samples taken during hypoglycaemia are few. Studying patients with subnormal blood glucose would be needed to be able to understand pathogenetic mechanisms from hypoglycaemia occurring in more common and clinically relevant stress situations, such as during acute illness or prolonged fast.

5.1.3. MITOCHONDRIAL DISEASES

Mitochondrial diseases are inborn errors of energy metabolism due to a defect in either mitochondrial (mtDNA) or nuclear DNA coding for proteins of the respiratory chain or other molecules needed for mitochondrial function (DiMauro and Schon 2003, Rahman and Hanna 2009). The most important function of mitochondria is oxidative phosphorylation, i.e. aerobic production of ATP energy in the electron transport chain; hence, some authors use the term mitochondrial diseases when referring only to disorders of oxidative phosphorylation (mutations in proteins of the electron transport chain). However, a broader definition such as the above, encompassing also other conditions with compromised mitochondrial function, is often used (DiMauro and Schon 2003). With a prevalence estimated between 1:5000 (Debray et al. 2008) and 1:10 000 (Shoffner 2001), mitochondrial diseases form an important group amongst inherited metabolic disorders.

Due to their heterogenous origin and the importance of mitochondria in almost all tissues, mitochondrial diseases have extremely variable clinical pictures with manifestations in different and often multiple target tissues. As mitochondrial diseases are, above all, disorders of energy metabolism, tissues with highest energy requirement, such as skeletal muscle, heart or liver, are most often affected. The most frequent clinical manifestation is that of a complex neurological or neuromuscular disease. However, symptoms may arise from any tissue and, depending on the particular mitochondrial disease, very specific tissues may be affected. This suggests that other, more complex mechanisms than simply a global failure to produce ATP energy are involved, but these mechanisms are currently poorly understood (DiMauro and Schon 2003).

The phenotype of those mitochondrial diseases caused by mutation in mtDNA is particularly varying due a phenomenon known as heteroplasmy. MtDNA has a high mutation rate, and as a result, the hundreds of mitochondria within a single cell may differ from each other – the cell is heteroplasmic (Wallace 1999). Following cell division, the wild-type and mutant-type mitochondria are distributed

differently to subsequent cells; often, a certain threshold number of pathologic mitochondria is needed to have clinical symptoms in a particular tissue. Due to the same phenomenon, also the inheritance pattern may vary, as mtDNA is maternally inherited – the symptoms of the progeniture depend on the percentage of mutant mitochondria they have inherited.

As the origin of the symptoms and the involvement of particular tissues in mitochondrial diseases is largely unknown, also their treatment remains supportive and symptomatic at best. None of the therapeutic approaches has been proven effective (Chinnery et al. 2006) with the only exception of a favourable response to coenzyme Q_{10} (ubiquinone) administration in primary coenzyme Q_{10} deficiency (Quinzii et al. 2007, Rahman and Hanna 2009).

5.1.4. HOMOCYSTINURIA

Homocysteine is derived from the sulphur amino acid methionine by S-adenosylmethionine – dependent transmethylation. Under normal circumstances, homocysteine may be further transsulphurated to cysteine or remethylated back to methionine (see 5.6.3; Mudd et al. 2001). The metabolic pathway for homocysteine production from methionine may exist in most cells and tissues, but the liver has the most central role for homocysteine production by transmethylation (Williams and Schalinske 2010).

Homocystinuria is an inborn error of metabolism in which homocysteine and its derivatives accumulate due to a metabolic blockage in these enzymatic pathways. Homocystinuria is most often caused by decreased or absent enzyme activity of **cystathionine β-synthase** (CBS, EC 4.2.1.22), which catalyses the first and irreversible step in homocysteine transsulphuration. The accumulating intracellular homocysteine is transported to blood; only a small proportion of plasma homocysteine then remains in the reduced, free, homocysteine form, and the rest forms homocystine disulphides or mixed disulphides with cysteine or protein sulphydryl groups (Mudd et al. 2001).

Homocystinuria due to CBS deficiency (OMIM 236200) is inherited as an autosomal recessive trait, but multiple underlying genetic defects exist, causing genetic and clinical heterogeneity. In addition, several rare defects in other enzymes participating to homocysteine metabolism have been identified, including several cobalamin-responsive forms of combined homocystinuria and methylmalonic acidurias (OMIM 277410; OMIM 277400; OMIM 277380) with defects in vitamin B12 (cobalamin) metabolism. The incidence of homocystinuria due to CBS deficiency has been reported to be 1: 344 000, making it a relatively rare inborn error of metabolism, but as the disease is inherited in a recessive autosomal manner, it is more frequent in some populations (Yap and Naughten 1998).

The clinical picture of homocystinuria due to CBS deficiency is varying, but patients often present starting from their second decade of life with mental retardation, skeletal abnormalities, ophthalmologic problems (dislocation of the lens, myopia) and thromboembolic vascular diseases (Mudd et al. 1985). Many CBS-deficient patients are responsive to pyridoxin (vitamin B_6) therapy; B_6 is needed as a cofactor for CBS, and responsive patients have some residual CBS activity that can be boosted with cofactor supplementation. In others, treatment consists of dietary restriction of homocysteine precursors and treatment of complications (Yap and Naughten 1998, Mudd et al. 2001).

5.2. ACQUIRED SITUATIONS WITH DISTURBED METABOLISM

Disturbances of metabolism can occur in other clinical situations than actual metabolic diseases. Metabolic problems typical of metabolic disease may occur in acquired conditions with secondarily disturbed metabolism in cells or tissues; for example, lactic acidosis may develop during critical illness following impaired peripheral circulation leading to compromised energy production. The well-coordinated metabolic balance between normal cells and tissues may be disrupted in the presence of malignant cells, which override metabolic control mechanisms. In addition, changes in the function or composition of certain metabolically active tissues, such as the liver or the intestinal bacterial flora, may have major metabolic consequences even if the original problem is not truly "metabolic".

Nutrition and metabolism are closely related. Problems in either one lead to perturbations in the other. In many inherited diseases of metabolism, nutrition is inadequate due to a metabolic blockage making normal nutrients harmful to the patients and causing further metabolic burden (e.g. protein, or branched-chain amino acid, intolerance in organic acidaemias). In a similar way, in certain other clinical conditions, metabolic problems may be due to an inadequate nutrition, either if nutrition is insufficient or if nutrients are poorly tolerated.

In this series of studies, cow's milk allergy (CMA) in early infancy has been used as a model of a situation where inappropriate nutrition leads to metabolic changes. CMA in infancy was chosen as it is a common clinical condition; in addition, as milk is the main nutrient in this age group, CMA may be seen a prototype of food intolerance. The possible metabolic changes in CMA could, then, be due to the infant's metabolic responses to poorly tolerated food or, as reviewed in 5.2.2, to the metabolic abnormalities of the altered intestinal microbiota in CMA.

5.2.1. COW'S MILK ALLERGY: GENERAL CONCEPTS

Cow's milk allergy is an immunological reaction to one or more of cow's milk proteins. It is often considered to be the prototype of food allergy, and it affects 2 – 2.5% of children during the first years of life in European countries (Mansueto et al. 2006) and in the USA (Sampson 2004); prevalences as high as 7.5% have been reported (Venter et al. 2006, Vandenplas et al. 2007). According to the underlying immunologic mechanism, CMA, as other food allergies, can be divided into IgE-mediated and non-IgE –mediated forms (Sicherer 2002), which also influences the diagnosis and prognosis. 60% of children with CMA have IgE-mediated reactions (Sampson 2004), and these tend to persist until later age; however, CMA is most often a temporary condition, as 80% of infants with CMA regain tolerance to cow's milk protein by the age of 3 years (Kneepkens and Meijer 2009).

The diagnosis of food allergies, including CMA, is challenging. No specific and sensitive diagnostic tests are available despite the high prevalence of these diseases. Especially during the first year of life, symptoms are often unspecific and general (Kneepkens and Meijer 2009), and almost half of the patients show a delayed type of reaction (Vandenplas et al. 2006), causing diagnostic confusion.

Several diagnostic procedures including skin prick tests (SPT) and allergen-specific IgE (sIgE) antibodies exist for IgE –associated CMA; however, they only measure sensitisation to the allergen and half of the sensitised children are not food allergic (Kneepkens and Meijer 2009). Thus, the laborious and resource-requiring double-blind placebo-controlled oral food challenge remains the gold standard (Sicherer 2002, Sampson 2004, Vandenplas et al. 2007, Kneepkens and Meijer 2009); it is also the only reliable diagnostic test for non-IgE –mediated CMA.

5.2.2. ALTERED INTESTINAL MICROBIOTA AND PERMEABILITY IN CMA

Intestinal flora is a tissue with substantial size and enormous metabolic activity (Nicholson et al. 2005); indeed, the 10^{14} bacteria of the human gut exceed 10-fold the number of cells in the human body (Penders et al. 2007), and its metabolic activity is equal to that of liver (Ouwehand 2007). Intestinal microbiota contribute to digestive and absorptive processes and their metabolites are also absorbed by the host: they fermentate undigested carbohydrates and non-digestable polysaccharides to short-chain fatty acids (SCFA), are involved in vitamin B (pantothenic acid, biotin, folic acid and vitamin B_{12}, or cobalamin) production and vitamin K synthesis (Guarner and Malagelada 2003, Guarner 2006). They also participate in ion and water absorption, and their metabolites affect cholesterol and bilirubin metabolism and some catabolic processes (degradation of beta-aspartylglycine, mucin etc.). In addition, bacterially produced SCFAs, especially butyrate, seem to have trophic

properties on the colonocytes of the intestinal epithelium (Salminen et al. 1998, Goossens et al. 2003, Nicholson et al. 2005).

5.2.2.1. Altered composition of intestinal microbiota

The intestinal microbiota is different in patients with food allergies as compared to healthy individuals (Bjorksten et al. 1999, Bjorksten et al. 2001, Kirjavainen et al. 2001, Kalliomäki et al. 2001a), Gore et al. 2008). Allergic children have higher counts of clostridia and less bifidobacteria in their faeces than do nonallergic children, and those of the allergic children with positive IgE-antibodies to food allergens appear to have even higher clostridia counts (Sepp et al. 2005). As the differences in intestinal microbiota precede the development of food allergies (Kalliomäki et al. 2001 a), Sepp et al. 2005), they do not seem to be completely secondary to the disease itself.

5.2.2.2. Altered metabolism of intestinal microbiota

Not only the composition but also the function, the metabolic activity, of intestinal microbiota is altered in atopic diseases (Norin et al. 2004, Ouwehand et al. 2007, Sandin et al. 2009). These changes precede allergic symptoms. Children with high risk for allergy had higher amounts of SCFA produced in bacterial metabolism in their faeces than do children with low allergy risk, and also their faecal SCFA profiles differed, there being less i-butyric and (i-) valeric acids in children later developing allergy (Kalliomäki et al. 2001 a), Norin et al. 2004, Sandin et al. 2009). In a similar way, in infants already having diagnosed allergy, the faecal SCFA composition showed less (i-) valeric, (i-) butyric and propionic acids, and higher levels of i-caproic acid (Böttcher et al. 2000).

In order to understand changes in intestinal microbiota, studying the metabolism of intestinal microbiota may be a more applicable method than identifying its composition by searching for different bacterial species. Less than 40% of intestinal bacteria can be cultured outside the gastrointestinal tract (Guarner and Malagelada 2003, Sandin et al. 2009), whereas the metabolites of the unculturable species may still be recognised.

5.2.2.3. Increased intestinal permeability

The intestinal inflammatory reaction secondary to the food allergy (Mansueto et al. 2006) causes changes in intestinal permeability by disrupting the intestinal epithelial barrier (MacDonald et al. 2005). Accordingly, children with food allergies

have been shown to have increased intestinal permeability to some compounds (e.g. lactulose) (Jackson et al. 1981, Laudat et al. 1994) and permeability to bacterial metabolites may well be increased in a similar way. Interstingly, there may be a two-way interrelationship between intestinal epithelial integrity and intestinal microflora, as it appears that the intestinal microbiota influence gut epithelial integrity (Isolauri et al. 2002, MacDonald and Monteleone 2005).

In summary, in a condition where there are concomitant changes in the composition and metabolic activity of the intestinal flora and in the intestinal permeability, measurable metabolic changes might also be shown in the host. Many end products of metabolism from human cells as well as from intestinal bacteria are excreted in the urine (Nicholls 2003, Nicholson et al. 2005, Walsh 2006). Following changes in intestinal permeability or intestinal microbiota, urinary excretion of end products of metabolism could thus be altered, which would offer new possibilities for the diagnosis of CMA or other food allergies; changes in intestinal microbiota have been shown to be reflected to urinary metabolite excretion (Nicholls 2003). Indeed, several studies have applied these findings to the diagnosis of food allergy (Laudat et al. 1994; Andre et al. 1987), but results from other studies are conflicting (Kuitunen et al. 1994; Catassi et al. 1995). In addition, it has been stated that only very small amounts of bacterial metabolites are noticeable in urine (Salminen et al. 1998)

Table 1. Mechanisms underlying possible metabolic changes in CMA.

References are marked with a superscript number in the table and listed in numeric order under the table.

PATHOLOGIC FEATURE ASSOCIATED WITH FOOD ALLERGY OR ATOPY	POSSIBLE INFLUENCE TO HOST METABOLISM
Intestinal inflammation with • Disruption of the intestinal epithelial barrier[1] • Increased intestinal permeability to some compounds[2, 3, 4, 5]	Permeability to normally unabsorbed compounds possibly reflected in urinary excretion of metabolic end-products
Different **composition** of intestinal microbiota [6] • More clostridia • Less bifidobacteria	Different microbiota produce different SCFA patterns Composition of intestinal microbiota influences intestinal epithelial integrity and absoption [12, 1]
Different **metabolic activity** of intestinal microbiota [7] • Elevated i-caproic acid in faeces [8] • Decreased propionic, (i)-butyric and (i)-valeric acids in faeces [8, 9, 10, 11]	Different SCFA patterns produced by intestinal microbiota • Noticeable in host metabolism[13] • Reflected in urinary excretion of metabolic end-products[13]

[1] MacDonald and Monteleone 2005; [2] Jackson et al. 1981; [3] Andre et al. 1987; [4] Dupont et al. 1989; [5] Laudat et al. 1994; [6] Sepp et al. 2005; [7] Ouwehand et al. 2007; [8] Böttcher et al. 2000; [9] Kalliomäki et al. 2001 a); [10] Norin et al. 2004; [11] Sandin et al. 2009; [12] Isolauri et al. 2002; [13] Nicholson et al.2005.

5.2.3. PROBIOTICS AND CMA

Probiotics are defined as live micro-organisms that, when administered in adequate amounts, have beneficial effects on the health and well-being of their host, regardless of their mechanism of action (Sanders 2008, Rijkers et al. 2010); this definition includes both commensal bacteria in the human gastrointestinal tract and genetically modified or biotechnology-derived species. As the use of probiotics in prevention and treatment of disease is the target of increasing scientific and commercial interest, even the definition of probiotics is in constant progress; earlier broader definitions included as well the products that these micro-organisms secrete (Paganelli et al. 2002), whereas originally, the term "probiotic" was used only to describe those micro-organisms and substances that had beneficial effects to the intestinal microbial balance (Fuller 1991). In therapeutic purposes, widely used probiotic strains include different species of lactobacilli (e.g. *Lactobacillus rhamnosus GG, Lactobacillus acidophilus, Lactobacillus reuteri*) and the *Bifidobacterium* species.

As the changes in gut microbiota in and preceding atopic diseases are becoming widely acknowledged, increasing interest has been drawn to the possible beneficial effect of probiotics in the treatment (Majamaa and Isolauri 1997, Viljanen et al. 2005, Weston et al. 2005, Brouwer et al. 2006, Taylor et al. 2007, Grüber et al. 2007) and in the prevention (Kalliomäki et al. 2001 b), Kalliomäki et al. 2003, Taylor et al. 2007, Kukkonen et al. 2007) of allergic diseases. This far, consensus has not been reached; the positive results obtained so far have also been questioned, and in some recent studies, probiotics have had no effect to atopic diseases (Matricardi et al. 2003, Brouwer et al. 2006, Taylor et al. 2007, Grüber et al. 2007). Some of the conflicting results may be explained by different study designs used, including different probiotic strains, different duration of the treatment and also the selection of study subjects. Immunologic mechanisms underlying allergic diseases are different and currently, it seems that probiotics are mainly effective in the treatment and prevention of IgE –mediated food allergies (Savilahti et al. 2008). In addition, it seems that their effect is, at best, moderate.

The exact mechanisms underlying the potential beneficial effects of probiotics in atopic diseases are unknown. It is generally thought that probiotics induce a low-grade inflammation in the gut and this, in turn, promotes tolerance to food allergens and downregulates allergic immune responses by inducing an immunologic shift favouring non-allergic Th1-type immunologic reactions, interleukin-10 and interferon-γ secretion (Savilahti et al. 2008). In addition, probiotics enhance intestinal barrier function, and some of their beneficial effects may be due to changes in the metabolic activity of intestinal microbiota following probiotic colonisation (Madsen et al. 2001, Rosenfeldt et al. 2004, Goossens et al. 2003).

Previous research has shown that the changes in the intestinal permeability following food allergies may be reversible (Dupont et al. 1989, Jalonen 1991), but it is unclear whether the disturbances in intestinal flora could also be reversible (e.g.

by probiotic treatment). It is known, however, that colonisation by probiotic strains after probiotic administration is transient, lasting only for weeks after cessation of probiotic treatment (Goossens et al. 2003, Savilahti et al. 2008).

Understanding the metabolic abnormalities associated to CMA and, eventually, the metabolic mechanisms of probiotic action in CMA could provide new approaches to the diagnosis and treatment of CMA.

5.3. REACTIVE OXYGEN SPECIES, OXIDATIVE STRESS AND ANTIOXIDANTS

5.3.1. CONCEPTS

Any chemical species capable of independent existence that has one or more unpaired electrons in an atom or molecular orbital is a free radical (Halliwell and Gutteridge 2007). As a single electron usually makes the species highly reactive and thus unstable, biologic molecules are mainly nonradicals. (Halliwell 1991). In biological systems, the hydroxyl radical (OH•) is the most reactive; it is capable of oxidising, i.e., receiving an electron from, most redox-active biomolecules.

Reactive oxygen species (ROS) are highly reactive metabolites of oxygen and some of them are also free radicals. Others, having no unpaired electrons, can be defined as nonradicals, but they are capable of high reactivity or free radical formation under special circumstances. An example of a nonradical reactive oxygen species is hydrogen peroxide (H_2O_2), which may form the extremely reactive hydroxyl radical in the presence of transition metals, such as iron.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\bullet + OH^-$$

The Fenton reaction: A hydroxyl radical is formed from the nonradical hydrogen peroxide in the presence of transition metals (here, iron).

An even broader concept is reactive species (RS), which encompasses not only oxygen-derived radicals and nonradicals, but also reactive nitrogen species (RNS) such as nitric oxide (NO•) and peroxynitrite ($ONOO^-$), as well reactive chlorine, bromine or sulphur species. Both ROS and RNS are known to play a role in human diseases; however, as some of the RNS, such as peroxynitrous acid (ONOOH) and

peroxynitrite ($ONOO^-$) are also metabolites of oxygen and could thus be classified as reactive oxygen species, the term ROS is often exclusively used in biomedical literature.

In the course of normal metabolism, ROS are continuously produced, and antioxidant systems (see 5.3.3) guard the organism against the oxidative damage that the reactive species are able to cause. Oxidative stress can be understood as an imbalance between these two counterparts, as an environment favouring oxidative reactions and deficient in reducing agents (Halliwell 2007). In principle, oxidative stress may result either from excessive free radical formation, impaired antioxidant defences, or both to some extent.

Reactions between ROS and biomolecules are part of normal metabolism; they serve multiple metabolic and regulatory purposes. ROS are involved in intra- and intercellular signalling through redox-regulated ion channels and transcription factors (Sen 1998), and they play a crucial role in regulation of cell survival and apoptosis via mitochondrial calcium release (Sen 1998, Halliwell and Gutteridge 2007, Decuypere et al. 2011). A moderate amount oxidative stress promotes survival in cell cultures (Burdon 1995), and in a similar way, an unusually oxidised environment is created in the endoplasmic reticulum on purpose to promote disulphide formation during protein folding (Halliwell 2000).

5.3.2. OXIDATIVE DAMAGE

When ROS are formed in excess, they damage biological structures by irreversibly oxidising them. All biomolecules are potential targets for oxidative damage, and some end products of different types of biomolecules subjected to oxidative damage may be measured in order to estimate oxidative damage (see 5.3.4).

As reducing a radical involves the transfer of an electron from another substance, which is, in turn, oxidised, these reactions have a tendency to continue as chain reactions. An example of this is lipid peroxidation, in which lipids are oxidised in a self-propagating reaction, giving rise to new lipid peroxide radicals.

5.3.3. ANTIOXIDANT SYSTEMS

An antioxidant is a substance that prevents or delays oxidation of its substrate, which can be almost any molecule found in living cells. An antioxidant is capable of this action even when present at low concentrations as compared to the substrate (Halliwell 1991; Halliwell 2007). Antioxidants can function either in the first line of defence by preventing the formation of reactive oxygen species, and making the already formed reactive oxygen species inactive – "scavenging" them – or, failing this,

by repairing the already produced oxidative damage (Halliwell 1990). In addition, the compartmentalisation of ROS-producing reactions inside the cells (such as the localisation of the enzymes of the electron transport chain in the mitochondrial membrane) can be viewed as an antioxidant mechanism. These mechanisms are summarised in **Table 2**.

Antioxidant systems mainly operate in collaboration with each other. Thus, superoxide dismutase is needed for disposal of superoxide, but the reaction produces hydrogen peroxide, which is removed by the action of catalase or glutathione peroxidase. To interrupt the chain reaction of lipid peroxidation (see 5.3.2), α-tocopherol, or vitamin E, is oxidised to α-tocopheryl radical, which is then reduced back to protective α-tocopherol by vitamin C, which is, in turn, recycled by glutathione (Sies 1991, Cheeseman and Slater 1993). It is important to notice that these examples are simplifications and result from *in vitro* evidence (Young and Woodside 2001, Halliwell 2007); in living organisms, antioxidant systems interact in a complex way to assure an optimal response to oxidative stress.

Table 2. Different mechanisms of antioxidant function (Halliwell 1991, Halliwell and Gutteridge 2007)

MECHANISM OF ACTION	EXAMPLE	FUNCTION
Prevention of or decrease in ROS production	Electron transport chain, cytochrome P 450	Compartmentalisation of ROS-producing reactions
Decreasing availability of oxidising agents	Transferrin Albumin	Binding of transition metal ions
Catalytic removal of ROS	Superoxide dismutase Catalase Glutahione peroxidase (GPx)	Disposal of superoxide Disposal of H_2O_2 Disposal of H_2O_2, other peroxides
Removal of ROS by being oxidised prior to more important molecules	Glutathione (GSH) α-tocopherol Ascorbic acid (vitamin C)	Oxidation of GSH to glutathione disulphide (GSSG) and reduction of radical
Restoration of antioxidant molecules	Glutathione reductase (GR) Glucose 6-phosphate dehydrogenase (G6PDH) Ascorbic acid and GSH	Reduction of GSSG to GSH Production of NADPH (reducing power for GR) Ascorbic acid restores α-tocopherol, and GSH restores ascorbic acid
Repair of oxidative damage	GSH, α-tocopherol DNA repair systems, e.g. • DNA glycosylase enzymes • Xeroderma Pigmentosum - proteins	Chain-breaking antioxidants in lipid peroxidation Base excision repair (removal of oxidatively damaged DNA bases) Nucleotide excision repair (removal of oxidative DNA lesions such as 8OHdG)

5.3.4. MEASURING OXIDATIVE STRESS

Oxidative stress, the imbalance between free radical production and antioxidant capacity, is a dynamic state prevailing in a biological organism. It is not possible to find a single and direct marker for oxidative stress. In principle, free radical formation and antioxidant systems could be studied separately to gain an understanding on their interrelationship. However, free radicals are, by definition, extremely reactive and short-lived species; techniques for their direct measurement in living systems by electron spin resonance have been developed, but are only of limited use. Radicals can also be detected by different techniques of radical trapping, which measure the more stable products of reactions between radicals and trap molecules, such as the formation of hydrogen peroxide (Abuja and Albertini 2001).

Antioxidant systems can be studied in a wide number of different tissues, but the interpretation of results may be problematic. A decrease in antioxidant levels can be due to an increase in its consumption, a decrease in its production, or both, and an increase in oxidative stress may either increase antioxidant levels or, when a extensive oxidative stress is present and cellular response mechanisms fail, decrease them. Antioxidants mainly function together and in successive reactions; measuring their steady-state concentrations only gives an idea of the net changes that have occurred. The consequence of depletion of a particular antioxidant participating in a chain reaction is not easily predictable. Additional complicating factors include differences in antioxidant systems and function between different tissues.

To solve these problems, techniques for measuring the overall antioxidant status in a particular tissue have been developed; such total antioxidant capacity assays measure the ability of a biological sample, e.g. a body fluid, to resist lipid peroxidation, and this ability is then compared to a known antioxidant (Abuja and Albertini 2001). In addition, expression of genes coding for proteins needed in antioxidant response can be studied, if relevant tissues are available for analysis.

A useful approach for understanding oxidative stress in living systems is to study oxidative damage, as this gives indirect evidence of either excess free radical formation or deficient antioxidant supplies. Identification of oxidatively damaged structures may also be clinically relevant in order to target therapeutic measures. The use of oxidatively damaged structures as markers of oxidative stress is summarised in **Table 3.**

Table 3. Oxidative damage to different cellular structures.
(DeZwart et al. 1999 and Halliwell and Gutteridge 2007)

GC Gas chromatography
HPLC High-pressure liquid chromatography
HPLC-ED High-pressure liquid chromatography with electrochemical detection
HPLC-MS High-pressure liquid chromatography and mass spectrometry

TARGET STRUCTURE	OXIDATIVELY DAMAGED STRUCTURE	EXAMPLE OF QUANTIFICATION METHOD
Lipids	Peroxidation of fatty acids, giving rise to	
	Aldehydes (e.g. MDA, HNE)	Detection of thiobarbituric acid –reactive species (TBA-RS) by spectrophotometry or HPLC
	Isoprostanes	Detection of F_2 –isoprostanes from arachidonic acid by GC- or HPLC-MS
	Exhaled hydrocarbons	Analysis (GC) of exhaled hydrocarbons (penthane, ethane)
Proteins	Carbonylated proteins	Carbonyl assay (reaction with 2,4-DNPH); most widely used
	Loss of thiol groups	Thiol group staining; Ellman's reagent
	Other changes to amino acids	e.g. HPLC detection of *o*-tyrosine
Nucleic acids (DNA)	Oxidised purine or pyrimidine bases	8-hydroxy-2'-deoxyguanosine assay
	Strand breaks	Incorporation of labelled bases, immunodetection of DNA fragments
	Deaminated purine or pyrimidine bases	8-nitroguanine or 5-guanidino-4-nitroimidazole measurement
	DNA-aldehyde adducts	GC-MS, HPLC-ECD –based quantification or immunoassay
	Gene-specific oxidative damage	Adherence to gene-specific probes after removal of oxidised bases

5.3.5. OXIDATIVE STRESS AND DISEASE: CAUSE OR CONSEQUENCE?

The toxic effects of oxygen have been known for centuries, but the concept of oxidative stress and the disease-causing potential of free radicals were first established when studying radiation injury (Gerschman et al. 1954). Half a century later, it was stated that oxidative stress, either in the form of excessive free radical production or impaired antioxidant defences, or both, occurs whenever cells and tissues are damaged (Halliwell and Gutteridge 2007). Oxidative stress has been associated to a wide range of acute or chronic disease states (e.g. retinopathy and respiratory distress syndrome in premature newborns, atherosclerosis, ischemia-reperfusion injury, neurodegenerative disorders, malnutrition), and the relationship of oxidative stress to aging has been intensively studied (Gil del Valle 2010, Seo et al. 2010, Decuypere et al. 2011).

This rather universal approach to oxidative stress has also been questioned (Sanz and Stefanatos 2008), since it is difficult to prove causality between oxidative stress and disease. Obviously, at some point of any disease process, cellular defence mechanisms against any harmful substance will fail; the coexistence of oxidative damage and a disease process does not necessarily indicate that oxidative stress has been a major causal factor throughout the development of the specific disease. Thus, when speaking about the interrelationship of oxidative stress and diseases, it may be more reasonable to concentrate on those diseases where oxidative stress has been shown to be present in earlier phases of the disease, thus truly contributing to the pathogenesis of complications.

5.3.6. INCREASED FREE RADICAL PRODUCTION IN METABOLIC DISEASES

Oxidative stress has been suggested to play a role in the pathogenesis of many metabolic diseases, such as the complications of diabetes mellitus (Abou-Seif and Youssef 2004, Gil del Valle et al. 2005), and in inborn errors of metabolism including mitochondrial diseases, hereditary tyrosinaemia, organic acidaemias (reviewed by Wajner et al. 2004), homocystinuria (Perna et al. 2003), phenylketonuria (Ercal et al. 2002) and maple syrup urine disease (Fontella et al. 2002). The mechanisms underlying increased free radical production in some inborn errors of metabolism are discussed below; the compromise of antioxidant systems and thiol metabolism are reviewed in 5.5.3.2 and in **Table 5**.

5.3.6.1. Organic acids interfere with mitochondrial ATP production

An increased free radical production following direct toxic effects of organic acids has been observed *in vitro*. Propionic and methylmalonic interfere with mitochondrial ATP production (Stumpf et al. 1980, Nakai et al. 1991, Dutra et al. 1993, McLaughlin et al. 1998, Okun et al. 2002) and disrupt intracellular ion homeostasis by inhibition of membrane Na+K+ATPase (Wyse et al. 1998, Wyse et al. 2000), resulting in an increase in free intracellular calcium, which favours free radical production (McLaughlin et al. 1998).

These mechanisms and the following exitotoxicity have been of special interest when explaining the neuropathology of organic acidaemias. Brain tissue is very susceptible to oxidative stress following its high metabolic activity and oxygen consumption, and high concentrations of transition metals and auto-oxidisable molecules including some neurotransmitters are present (Wajner et al. 2004). Accordingly, many previous studies have associated other neurodegenerative disorders to mitochondrial dysfunction, energy failure, oxidative stress and

exitotoxicity acting in a synergistic way (reviewed by Wajner et al. 2004). With further research on oxidative damage and antioxidant status in organic acidaemias, similar mechanisms could well account for some of the unexplained neurological symptoms in these diseases.

The development of neurological problems in organic acidaemias has thus been studied to some extent. The pathogenesis of episodes of metabolic decompensation, the "metabolic crises", that occur in organic acidaemias and have a major influence to the patients' prognosis, is less clear. For instance, patients with organic acidaemias often present with pancreatitis, but the precipitating factors are not understood (Kahler et al. 1994). Interestingly, oxidative stress has been suggested to play a role in the development of pancreatitis in animal models (Schoenberg et al. 1995, Rau et al. 2000) and in humans (Schoenberg et al. 1995, Park et al. 2003). In addition, deficiencies of antioxidant systems, including glutathione, have been noticed in association with pancreatitis (Rau et al. 2000, Park et al. 2003). Some patients with pancreatitis even respond to antioxidant therapy (Schoenberg et al. 1995, Schulz et al. 1999), even if this issue remains unclear (Mohseni Salehi Monfared et al. 2009). Thus, pancreatitis and oxidative stress might account for some of the episodes of metabolic decompensation in patients with organic acidaemias. This could also provide a target for treatment with antioxidants (Treacy et al. 1996).

5.3.6.2. Lack of energy and reducing power in hypoglycaemia lead to mitochondrial dysfunction and excitotoxicity

Under normal conditions, the production of free radicals is controlled and matches antioxidant supplies. When cellular energy metabolism is compromised, as occurs during hypoglycaemia, control mechanisms may fail and the production of free radicals in mitochondria and in cellular stress responses increase. Indeed, in experimental settings, hypoglycaemia, or glucose deprivation, has been associated with an increase in free radical production. In these studies, experimental animals have reached profound insulin-induced hypoglycaemia with blood glucose 1 mmol/l (McGowan et al. 2006) or with isoelectric EEG (Ferrand-Drake et al. 1999, reviewed by Auer 2004, Suh et al. 2008); alternatively, cultured cells have been totally deprived of glucose with no glucose in the cell culture medium (Coleman et al. 2007, Suh et al. 2007) or chemical inhibition of glycolysis (Rego et al. 1999).

The increase in free radical production during these rather extreme conditions has been related to a failure to maintain vital cellular control mechanisms. With depletion of energy supplies (Auer 2004) and lack of reducing power following inhibition of glycolysis (Rego et al. 1999) in profound hypoglycaemia mitochondrial dysfunction follows. This leads to "leakage" of mitochondria, an increase in

mitochondrial superoxide and hydrogen peroxide generation (McGowan 2006). In addition, cellular stress responses are activated, resulting in excitotoxicity and activation of cell death processes. Activation of neuronal glutamate receptors (Wieloch 1985, Suh 2007), increased intracellular calcium levels (Coyle and Puttfarcken 1993, Singh 2004), mitochondrial permeability transition (Ferrand-Drake et al. 1999), altered redox status of the cell (Moley and Mueckler 2000) and activation of free radical –producing enzymes such as NADPH oxidase producing superoxide (Suh 2007) are crucial elements in a cascade causing increased ROS formation and ultimately leading to hypoglycaemic cell death.

5.3.6.3. Dysfunctional mitochondria overproduce reactive species

Mitochondria are a major source of ROS even under physiologic circumstances; 1-2% of the molecular oxygen consumed in mitochondrial oxidative phosphorylation is metabolised to ROS following electron leakage from the respiratory chain. However, compromised function of the electron transport chain (ETC) has been shown to lead to an overproduction of reactive oxygen species (ROS) (Esposito et al. 1999, Wallace 1999, Chen et al. 2003).

Accordingly, in patients with mitochondrial diseases (Wani et al. 2008) or in cultured cells with dysfunction of the mitochondrial respiratory chain (Pitkänen and Robinson 1996, Luo 1997), increased ROS production – measured as either increased hydroxyl radical or superoxide levels - has been observed.

The overproduction of ROS by poorly functioning mitochondria is particularly detrimental, as ROS exposure has been shown to further compromise mitochondrial function by disrupting mitochondrial membranes and inactivating the iron-sulphur centres in complexes I, II and III of the electron transport chain (Bolanos et al. 1996, and reviewed by Wallace 1999). Mitochondrial dysfunction and increased ROS production thus seem to form a vicious cycle with one worsening the other.

5.3.7. ANTIOXIDANT THERAPY

The amount of ROS production in a living organism is not readily influenced. In order to protect cells and tissues agains oxidative stress, the most logical therapeutic approach is to increase the amount of antioxidants available. Indeed, antioxidant therapy, and antioxidants in primary or secondary prevention of disease, has been the focus of innumerable studies. However, irrespective of the studied disease or antioxidant, nearly all of the clinical trials have had disappointing results (Chinnery et al. 2006, Steinhubl 2008, Bjelakovic et al. 2010, Bjelakovic et al. 2011). It seems

that the function and complex interplay between antioxidant systems in living organisms is not known to a sufficient degree to be able to target antioxidant therapy when it would be needed (Bjelakovic et al. 2011).

As an exception to these unpromising results, in some situations where there is a selective deficiency of a specific antioxidant, antioxidant therapy has been shown to be effective. Examples of these include the treatment of severe GSH depletion in paracetamol (acetaminophen) intoxication with N-Acetylcysteine (Hinson 2010), which is a precursor of cysteine, the rate-limiting substance for GSH synthesis; or the supplementation of coenzyme Q_{10} (ubiquinone) to patients with a mitochondrial disease caused by primary coenzyme Q_{10} deficiency (Quinzii et al. 2007).

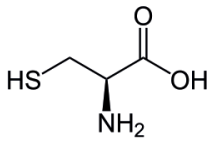
Possibilities for thiol antioxidant treatment are reviewed in section 5.5.4.

5.4. THIOLS

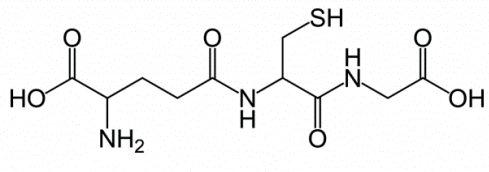
5.4.1. STRUCTURE

A thiol is a compound with a sulphydryl (-SH) group, also known as a thiol group, as the functional group. An aminothiol is a thiol associated to an amino acid. In humans, aminothiols are found in proteins and in other chemical structures, also known as non-protein thiols. The amino acid cysteine (CYSH, see also 5.4.2) is the predominant aminothiol in the extracellular compartment, whereas intracellularly, the tripeptide glutathione (γ -glutamyl-cysteinyl-glycine, GSH; see also 5.4.3) predominates (Jones et al. 2000). Glutathione may be oxidised with itself to form glutathione disulphide (GSSG), or with protein sulphydryl groups, forming mixed disulphides. In a similar way, cysteine is oxidised to cystine (CYSS), which contains a disulphide bond between two oxidised cysteine molecules.

In addition to cysteine and glutathione, **homocysteine**, which is an intermediate in sulphur amino acid metabolism and a precursor of cysteine (see 5.4.2 and 5.4.3), participates to thiol metabolism. Homocysteine is able to form mixed disulphides with cysteine and glutathione as well as with protein sulphydryl groups, and is part of plasma thiol pool (Ueland et al. 1996). Normally, plasma homocysteine levels are low as compared to cysteine levels, but in patients with homocystinuria or in some other pathologic conditions associated to high homocysteine levels, plasma homocysteine significantly participates in extracellular thiol-disulphide reactions and influences thiol redox status (Di Giuseppe et al. 2004).



Cysteine (CYSH)



Reduced glutathione (GSH)

5.4.2. CYSTEINE

Cysteine is an amino acid with a sulphydryl group, which allows it to form disulphides either with itself (yielding cystine) or with other thiols. Cysteine is not an essential amino acid in humans, as it can be synthesised from methionine, another sulphur-containing amino acid. The transsulphuration pathway converting methionine to cysteine only takes place in the liver (Lu 1998) and consists of five sequential enzymatic reactions.

In addition to participating to protein synthesis as a nonessential amino acid, cysteine is also needed to produce non-protein structures, including glutathione, taurine and coenzyme A (Stipanuk et al. 2006). Cysteine is considered to be the rate-limiting substrate for GSH synthesis (Deneke and Fanburg 1989, Lu 1998). Besides the synthetic processes, cysteine also has regulatory roles. Under physiological conditions, cysteine and methionine are the only amino acids showing reversible redox changes and thus participating to redox regulation of cellular functions (Blanco et al. 2007). As a result, both intra- and extracellular cysteine levels are tightly regulated and kept at a narrow range even when dietary cysteine intake varies (Stipanuk et al. 2006).

Some controversy has prevailed over the question whether the transsulphuration pathway is active already in the neonatal period and, hence, whether cysteine might be an essential amino acid for newborns (Levonen et al. 2000, Riedjik et al. 2007, Thomas et al. 2008). In addition, even in older children and adults, cysteine may be considered ”conditionally essential” (Tesseraud et al. 2009); the need for cysteine may exceed the transsulphuration capacity of the liver under stress conditions such as acute illness, when up to 40% of the increased cysteine need appears to be due to an increase in GSH synthesis (Metayer et al. 2008). Thus, a sufficient dietary intake of both methionine and cysteine seems to be needed to assure adequate GSH synthesis (Bray et al. 1993, Wu et al. 2004, Metayer et al. 2008), thereby coupling dietary protein intake, nutritional status and thiol homeostasis (see 5.4.3 and 5.5.3.1).

5.4.3. GLUTATHIONE: STRUCTURE, SYNTHESIS AND ITS REGULATION BY CYSTEINE AVAILABILITY

Glutathione is a tripeptide formed from glycine, glutamate and cysteine with a γ-carboxyl linkage between the glutamate and cysteine moieties (Hopkins 1929, Harington and Mead 1935). Glutathione is present in virtually all eukaryotic cells in the millimolar range (Meister and Tate 1976). It is made in two consecutive ATP-consuming reactions from glycine, glutamate and cysteine catalysed by γ-glutamylcysteine synthetase (GCS; EC 6.3.2.2), also called glutamate-cysteine ligase, and glutathione synthetase (GS; EC 6.3.2.3) (Meister and Anderson 1983) (**Figure 2**). The γ-carboxyl linkage between glutamate and cysteine is important for GSH function, as it accounts for the intracellular stability of GSH (Meister and Tate 1976): only γ-glutamyl transpeptidase, also called γ-glutamyl transferase (γGT; EC 2.3.2.2), located on extracellular surfaces, is able to hydrolyse the bond. GCS activity is rate limiting for GSH synthesis, and the enzyme is feedback inhibited by GSH (Meister and Anderson 1983, Stipanuk et al. 1992).

Cysteine is often considered the rate-limiting substrate for GSH synthesis (Deneke and Fanburg 1989, Lu 1998), as in plasma, glycine and glutamate are normally found in abundance (Stein and Moore 1954) as compared to more limited cysteine supplies. Moreover, the K_m value of GCS for cysteine is close to intracellular cysteine levels, whereas intracellular glutamate levels clearly exceed the K_m of GCS for glutamate (Lu 1998). Under special circumstances (e.g. in preterm babies, during foetal growth, in patients with sepsis, systemic inflammation or intense haemolysis, and during inadequate protein intake), however, glycine supplies may become a limiting factor for GSH synthesis (Jackson et al. 1987, Wu et al. 2004, Jackson et al. 2004).

It seems that cysteine availability mainly controls the rate of GSH synthesis, although additional control mechanisms exist, as GSH itself feedback inhibits GCS activity (Meister and Tate 1976, Meister and Anderson 1983). In humans, GSH synthesis rates have been shown to increase when cysteine or its precursors are supplemented in various situations with low GSH levels (Wu et al. 2004, Badaloo et al. 2002), and blood GSH synthesis rates (but not GSH levels) diminish when sulphur amino acid intake is inadequate (Lyons et al. 2000). Cysteine supplementation has been shown to lead to increased blood or intracellular GSH levels in experimental animals (Tesseraud et al. 2008; Stipanuk et al. 2006). Even during low cysteine intake, utilisation for GSH synthesis has a priority in cysteine metabolism (Stipanuk et al. 1992).

The nutritional aspects of cysteine intake and GSH synthesis are discussed in more detail in 5.5.3.1.

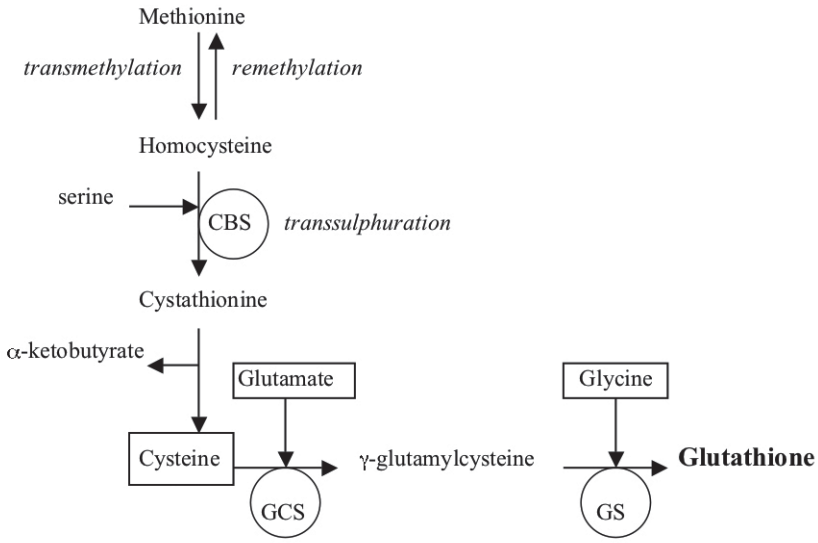


Figure 2. Glutathione synthesis and cysteine precursors.

All the intermediates are not represented. Transmethylation of methionine to homocysteine is confined to the liver, whereas GSH synthesis from its precursors glutamate, cysteine and glycine takes place in virtually all tissues. The sequential conversion of methionine to cysteine via homocysteine, the transsulphuration pathway, involves several steps (all are not represented).
CBS Cystathionine β-synthetase
GCS γ-glutamylcysteine synthetase
GS Glutathione synthetase

5.4.4. GLUTATHIONE FUNCTION

Glutathione is present in virtually all tissues and has multiple essential functions. Glutathione is, above all, involved in protection against toxic and harmful compounds, but it serves also other important purposes.

5.4.4.1. Glutathione in antioxidant reactions

GSH is an important intracellular antioxidant with a millimolar concentration. To scavenge (reduce) reactive oxygen species and peroxides, reduced glutathione can form disulphides with itself (oxidised glutathione i.e. glutathione disulphide, GSSG), or with other thiol compounds, including protein thiol groups (mixed disulphides). The oxidation of GSH is catalysed by different isoforms of glutathione peroxidase (GPx), although also non-enzymatic reactions are possible. Glutathione reductase (GR) can reduce GSSG back to protective GSH. The reducing power comes from NADPH formed in the pentose phosphate route where glucose-6-phosphate dehydrogenase (G6PDH), is the first and rate-limiting enzyme (**Figure 3**).

Figure 3. The glutathione redox cycle

GSH reduced glutathione
GSSG oxidised glutathione (glutathione disulphide)
GPx glutathione peroxidase
GR glutathione reductase
G6PDH glucose 6-phosphate dehydrogenase
ROOH peroxide
ROH reduced peroxide
To reduce peroxides, GSH is oxidised to GSSG. The reaction is catalysed by GPx. GSH is restored, when GR catalyses the reduction of GSSG back to GSH. For the function of GR, reducing power from NADPH is needed. NADPH is regenerated in the pentose phosphate route, where the activity of G6PDH is rate-limiting. (Modified from Meister, 1988 and Meister, 1995).

5.4.4.2. Glutathione in conjugation reactions

Glutathione is involved in the detoxification of many compounds, which can be excreted as soluble glutathione conjugates, or be further degraded by γ-glutamyltranspeptidase; the remaining cysteine conjugate is metabolised to mercapturic acid, which is excreted to urine. **Glutathione transferase** (GST) enzyme superfamily, formerly called the glutatione-s-transferases (Mannervik et al. 2005), catalyses the conjugation reactions between GSH and xenobiotics. In the conjugation reaction, the glutathionyl group (GS-) from glutathione is transferred to an electrophilic molecule (Whalen 1998, Mannervik 2005). Different GST enzymes may be divided into **soluble GSTs** encountered mainly in the cytosol (but also in organelles including mitochondria) and to **mitochondrial GSTs** operating inside mitochondria, and, finally, to **microsomal GSTs** which are integral membrane proteins (Mannervik 2005). The transcription of GST enzyme family is regulated by the same antioxidant response element (ARE) that also regulates γGCS and other antioxidant enzyme expression (Itoh et al. 2004); thus, the two protective functions of GSH - conjugative and antioxidative - are coupled.

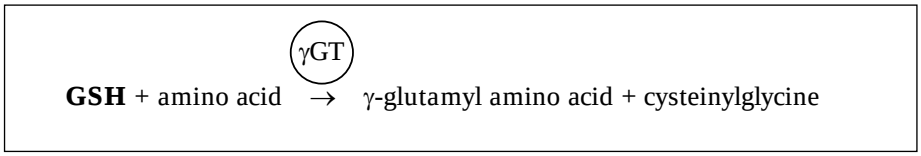
5.4.4.3. Glutathione in cysteine storage and transport

In the extracellular compartment, glutathione acts as a reservoir and transporter of cysteine as it is less prone to oxidation than cysteine is. Circulating glutathione can be cleaved to yield cysteine by γ-GT on external surfaces of cellular membranes (Meister and Tate 1976). The ability of diverse cell types to export GSH may also be related to this function of glutathione; GSH efflux provides other cells with cysteine (Meister and Anderson 1983), GSH only acting as a more stable transport form.

Also kinetic studies with stable isotopes show that the endogenous disappearance rate of GSH is responsible for the majority of whole body cysteine flux (Lyons et al. 2000), which further supports the idea of GSH functioning as a transport form of cysteine (Wu et al. 2004).

5.4.4.4. Glutathione in amino acid transport; the γ-glutamyl cycle

Tissues with high γ-GT activity (e.g. renal tubular cells, lung type II alveolar cells, intestinal epithelial cells) use a common transport mechanism for amino acid and GSH uptake from blood (Meister and Tate 1976, Meister and Anderson 1983). The tripeptide GSH and an amino acid are cleaved by γ-GT which is an integral membrane protein located on the extracellular surface.



The resulting two dipeptides (γ-glutamyl-amino acid and cysteinylglycine), may then be used for intracellular protein and GSH synthesis. Cysteinylglycine is further degraded extracellularly by **dipeptidase** to cysteine and glycine, which are then uptaken by the cell. The γ-glutamyl-amino acid moiety, in turn, is degraded intracellularly by **γ-glutamyl cyclotransferase** to oxoproline, which is further metabolised to glutamate by **5-oxoprolinase**. Thus, all three parts (glutamate, cysteine and glycine) needed for intracellular GSH synthesis have been reconstituted.

The degradation of GSH on extracellular surfaces, followed by the uptake of amino acids by the cell and intracellular GSH synthesis is called the **γ-glutamyl cycle**. It is an important mechanism for regulating interorgan GSH homeostasis, as explained in 5.4.5.; in addition, it couples GSH to yet another vital cellular function, amino acid uptake.

5.4.4.5. Role of glutathione and intracellular thiol redox status in redox signalling and regulation

Many intracellular signalling cascades, including enzymes, receptors, transport proteins or transcription factors are redox-regulated through oxidation and reduction of sulphydryl groups in protein cysteine residues (Dalle-Donne et al. 2007, Mieyal et al. 2008). Proteins can be directly redox-regulated through

conformational changes following oxidation or reduction of their sulphydryl groups. Redox control can also be exerted via redox-sensitive transcription factors, such as NF-κB, which are activated by reactive oxygen and nitrogen species (Ballatori et al. 2009 b). Glutathione is the most abundant intracellular non-protein thiol, and it is able to form mixed disulphides with protein thiol groups. This reversible process, called protein **S-glutathionylation**, is an important posttranslational modification and cellular regulatory mechanism, and it seems to function both as a response to oxidative stress (Fratelli et al. 2005) and under physiological conditions (Dalle-Donne et al. 2007). Thus, GSH has an important role in the maintenance of intracellular redox potential and the function of redox-sensitive control mechanisms, and this far, the multiple roles GSH serves in these critical processes are only being elucidated (Sabens et al. 2011).

5.4.4.6. Glutathione as a possible neuromodulator or neuroprotective agent

Glutathione has an important role as an antioxidant in the central nervous system, which is responsible of 20% of the oxygen consumption of the human body and thus a major source of ROS; indeed, GSH is critical for neuronal survival (Ballatori et al. 2009, b). However, it has been suggested that glutathione may also have other neuroprotective and neuromodulative roles, and that it may even act as a neurotransmitter (these hypotheses are reviewed by Janáky et al. 1999, Ballatori et al. 2009, b). It seems that GSH is both a ligand and a modulator of the glutamate NMDA receptor (Janáky et al. 1999, Oja et al. 2000, Ballatori et al. 2009, b), and that there may even be specific "GSH receptors" in the central nervous system (Janáky et al. 1999).

5.4.5. INTERORGAN HOMEOSTASIS OF GLUTATHIONE; THE ROLE OF PLASMA AND ERYTHROCYTE THIOLS

5.4.5.1. Interorgan, intercellular and intracellular glutathione homeostasis

Most tissues and cell types are able to synthesise GSH. However, liver is essential for supplying GSH to the whole organism for two reasons: first, only liver can synthesise cysteine from methionine by the transsulphuration pathway (Lu 1998), thus providing the rate-limiting component for GSH synthesis, and, second, hepatocytes export GSH in large amounts to be then taken up into tissues with high γ-GT activity, e.g. renal tubular cells or cells in jejunal villi (Meister and Anderson1983). In addition, hepatocytes also secrete GSH to bile; GSH is released to the intestine and its constituent amino acids are reuptaken in the enterohepatic cycle. Also

other tissues are able to export GSH (Ballatori et al. 2009 a), but the role of liver is essential in supplying other tissues with GSH (**Figure 4**).

Glutathione is a hydrophilic molecule and cannot pass freely through cellular membranes. The transport of GSH from plasma to cells is coupled to amino acid uptake, and it mainly occurs via extracellular GSH cleavage to cysteinylglycine and γ-glutamyl-amino acid moieties by γ-glutamyl transpeptidase. These dipeptides can then be used for intracellular GSH synthesis and/ or protein synthesis (see 5.6.4). The activity of γ-glutamyl transpeptidase determines the extent to which different cell types are able to import GSH from the extracellular space (i.e., from plasma, intestinal lumen etc.) (Meister and Tate 1976). Kidneys, with very high γGT activity, are responsible for most of GSH turnover (Lash 2009) in the body. Hepatocytes, in turn, have virtually no γGT activity, whereas in biliary epithelium, γGT activity is high. This reflects the liver's central role in supplying GSH to other tissues rather than taking it up for its own use. In addition to the γGT-dependent GSH transport mechanism, kidneys seem to be able to take intact GSH from cell to cell, or from the extracellular space to the cell, across the basolateral membrane (Lash and Jones 1984 and 1985, Lash 2009). This phenomenon has been a subject of debate (Ballatori et al. 2009 a), Lash 2009) and its physiological significance, as well as the exact structure of GSH transporters serving this purpose, remains unknown (Lash 2009).

In contrast to GSH import, the mechanisms of GSH export from cells are better understood. Some of the multidrug resistance –associated proteins (Mrp; Mrp-1 and Mrp-2, possibly Mrp-4 and Mrp-5) have been identified as GSH transporters, and they are also able to export GSSG and glutathione S-conjugates (Ballatori et al. 2009, a). GSH and GSSG export through Mrp and, probably, other transporters, is an important mechanism for controlling intracellular thiol redox status and cell survival. Excess GSSG is exported during increased oxidative stress to maintain adequate GSH/GSSG balance (Jones 2002) and GSH export, in turn, may induce thiol redox changes triggering apoptosis (Hammond et al. 2007).

In addition to interorgan and intercellular homeostasis mechanisms, GSH is compartmentalised intraracellularly. Mitochondria have a separate GSH pool needed for local protection against ROS produced in oxidative phosphorylation. At least two different carrier proteins (the dicarboxylate and the 2-oxoglutarate carriers) transport cytosolic glutathione as intact GSH across the inner mitochondrial membrane (Lash 2006). Mitochondrial GSH status is an important determinant of mitochondrial function and ROS production, which further participate to the regulation of cell viability and signalling (Fernandez-Checa et al. 1998, Lash 2009).

Knowledge on GSH synthesis and levels in different tissues *in vivo* is incomplete, as GSH metabolism is complex and involves intracellular, intercellular and interorgan compartmentalisation (Wu 2004). Only fairly recently, the use of stable isotopes in order to study *in vivo* rates of GSH synthesis has given new information about GSH metabolism on the scale of the whole organism (Lyons et al. 2000, Wu et al. 2004).

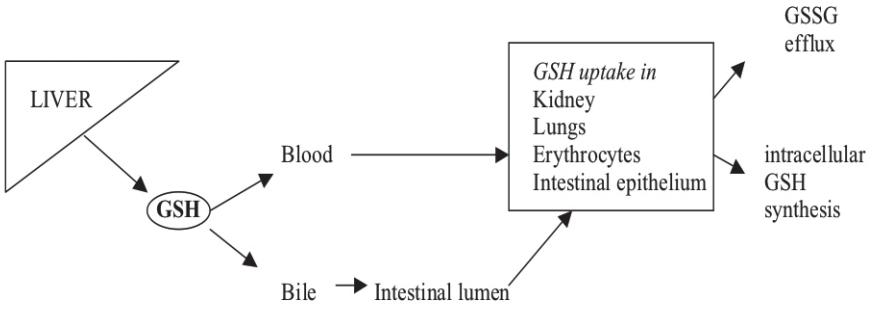


Figure 4. Interorgan transport of GSH.

The liver is able to synthesise cysteine and to export GSH. The liver provides other tissues with glutathione (and cysteine) via GSH release to plasma and bile.
The uptake of GSH in different tissues mainly depends on their gGT activity. GSH is degraded to its constituents for uptake and then resynthesised intracellularly in extrahepatic tissues. In order to maintain their GSH/GSSG redox status, cells may export GSSG.
Plasma GSH mainly arises from the liver. Sources of plasma GSSG also include extrahepatic tissues such as erythrocytes and lungs that release GSSG into the circulation under oxidative stress.

5.4.5.2. Plasma glutathione: relevance, sources and function

Glutathione is predominantly an intracellular antioxidant and detoxifier; plasma GSH levels are very low, in the micromolar range (1-10 μmol/l) (Halliwell 1990). Although more stable than cysteine, also plasma GSH is relatively short-lived, with a half-time of a few minutes (Jones et al. 1998), and thus efficient uptake to cells by extracellular γGT activity is essential. Due to these characteristics, the analysis of plasma GSH is technically demanding. Even minor haemolysis during blood sampling or sample procession leads to an artefactual increase in plasma GSH levels, as erythrocyte GSH levels exceed plasma levels 500-fold, and immediate treatment of the sample is needed to avoid GSH oxidation.

Glutathione antioxidant enzyme activity is present also in human plasma, but it seems that plasma GSH levels are too low to truly participate to antioxidant defence in the extracellular compartment (Halliwell and Gutteridge 1990). Instead, the function of plasma glutathione seems to be related to amino acid transport and to the maintenance of extracellular thiol status.

Plasma GSH largely arises from the liver (Meister 1988, Ookhtens and Kaplowitz 1998), although many cell types release GSH to the extracellular space following cellular damage. This may further trigger apoptotic pathways by influencing intracellular thiol redox status (van den Dobbelsteen et al. 1996, Hammond et al. 2007, Ballatori et al. 2009 a). In addition, under oxidative conditions, GSSG is actively transported from cells in order to maintain intracellular thiol redox balance (Jones 2002, Ballatori et al. 2009 a). GSH and GSSG export are mediated by the multidrug resistance –associated proteins (Mrps) and, possibly, other transporters

(Ballatori et al. 2009 a). This contributes to plasma total glutathione levels and plasma GSH/GSSG redox status.

Thus, plasma GSH level reflects a complex interorgan metabolism and plasma GSH, as such, is certainly not an adequate measure of antioxidant defences or oxidative stress. Plasma GSH levels, however, probably reflect intracellular glutathione levels available for antioxidant defence as GSH release from cells depends on its intracellular concentrations (Jones 2002). Consequently, it has been postulated that plasma glutathione may represent glutathione levels in less accessible tissues (Pastore et al. 2003) and be an indicator of long-term GSH status in the organism (Taylor et al. 1992). Plasma GSH levels, however, represent the net sum of interorgan GSH metabolism, and changes in plasma GSH may not always be a reliable measure of GSH levels in cells or other tissues (Jones et al. 1998, Wu et al. 2004).

Additional factors such as dietary uptake of sulphur amino acids, fasting, age and sex also influence plasma GSH levels to some extent (Blanco et al. 2007), although tight metabolic control exists to minimise changes in these crucial components of plasma thiol redox status (Stipanuk et al. 2006).

In summary, plasma GSH appears to arise mainly from the liver. Sources of plasma GSSG include also extrahepatic tissues such as erythrocytes and lungs that release GSSG into the circulation under oxidative stress (Adams 1983). Plasma GSH/GSSG ratio could then, to some extent, express the balance between antioxidant capacities (liver GSH release) and the need for GSH in oxidative reactions (tissue GSSG export).

5.4.5.3. Erythrocyte glutathione

As oxygen carriers, erythrocytes are continuosly exposed to high oxygen concentrations and thus susceptible to oxidative damage. Moreover, as anucleate cells, their ability to repair damaged proteins and lipids is limited. To face these challenges, erythrocytes are rich in antioxidant systems, including GSH and enzymes needed for its synthesis and antioxidant function (Kurata 1993). In order to maintain intracellular redox balance, erythrocytes excrete GSSG by an ATP-dependent active mechanism under situations of increased oxidative stress and thus contribute to plasma GSH/GSSG ratios (Lunn et al. 1979, Adams et al. 1983).

In vivo studies on GSH kinetics have shown that erythrocytes have a high turnover rate for GSH, and indeed, in healthy human subjects, erythrocytes completely replace their GSH in 1.5 days, which represents nearly 10% of the GSH synthesis in the whole body (Lyons et al. 2000). Interestingly, even if erythrocytes have high GSH synthesis rates, they lack γGT activity (Srivastava 1976) and are thus unable to use the conventional way of extracellular GSH degradation for intracellular GSH

synthesis. Instead, erythrocytes transport the constituent amino acids cysteine, glycine and glutamate (in the form of glutamine) by specific sodium-dependent transporters (Raftos et al. 2010). Thus, erythrocytes are dependent on continuous amino acid supply for their antioxidant defences, and seem not to be able to profit from GSH supply from the liver.

5.4.5.4. Plasma cysteine and other plasma aminothiols

Reduced cysteine is the predominant low-molecular weight thiol in the extracellular compartment, including plasma (Ueland et al. 1996). Reduced cysteine, however, is only a small part of the total plasma cysteine pool, which includes reduced cysteine (3-4% of the total plasma cysteine pool), free oxidised cysteine (cystine, CYSS; or cysteine in mixed disulphides with other non-protein thiols, including GSH and homocysteine; 30% of total) and protein-bound cysteine (65% of total) (Ueland 1995, Ueland et al. 1996).

Plasma cysteine has an important role in metabolic and regulatory processes by serving as a precursor for glutathione and being able to participate in redox reactions (see 5.4.6). As a result, the levels of plasma cysteine are tightly regulated, and excess cysteine is toxic. Also intracellular cysteine levels are subject to regulation, but the extracellular levels are kept at an even narrower range, varying from 80 to 220 or 250 μmol/l (Ueland et al. 1996, Stipanuk et al. 2006). The liver has a central role for regulating plasma cysteine levels. This is also coupled to the liver's ability to control GSH levels, GSH serving as a cysteine reservoir. By controlling the synthesis of cysteine from methionine, the synthesis of GSH from cysteine and the differential metabolic fates of cysteine (including synthesis of coenzyme A, taurine and inorganic sulphate), the liver acts a buffer against variations in plasma cysteine levels following changes in dietary cysteine intake (Stipanuk et al. 1992, Bella et al. 1999, Stipanuk et al. 2006).

Plasma cysteine, plasma cysteinylglycine and plasma homocysteine in their reduced, free oxidised ("non-protein disulphide") and protein-bound forms comprise the plasma redox thiol status, a dynamic system participating in regulatory processes and influencing intracellular functions (see 5.4.6). Of these, under normal conditions, plasma cysteine pool is by far the most important, as it exceeds the levels of homocysteine and cysteinylglycine pools about 20- and 10-fold, respectively (Ueland et al. 1996). When levels of homocysteine are elevated, also plasma homocysteine pool may be an important factor in determining extracellular redox status.

5.4.6. THIOL REDOX STATE

5.4.6.1. Reduction potential, E_h

In order to describe the reductive or oxidative properties of GSH/GSSG or CYSH/CYSS pool, which influence many cellular functions as described in 5.4.4, their electrochemical reduction potential (E_h) may be calculated, using the standard reduction potential (E_0) for each reduction/ oxidation (redox) pair.

In general, a more negative E_0, expressed in millivolts, indicates that the redox pair in question will, under standard conditions, be oxidised prior to one with a more positive E_0. The "actual" reduction potential E_h is calculated from the E_0 by the Nernst equation. It takes into account the ratio of the concentrations of the reduced and the oxidised pair of the couple and the temperature during the specific circumstances in question.

However, as in humans *in vivo*, temperature is fairly constant, instead of calculating redox potentials, it is informative to use only the ratio of concentrations of GSH to GSSG or CYSH to CYSS in order to express their redox state (Adams et al. 1983). This approach is simpler and useful, but it does not allow direct comparison between different redox pairs (Jones et al. 2000).

5.4.6.2. Role of thiol redox state: intra- and extracellular redox regulation and signalling

Under physiological conditions, sulphur amino acids, i.e. extracellular cysteine/cystine and intracellular GSH with its cysteine moiety, are the only amino acids that participate in reversible redox reactions (Blanco et al. 2007). These are essential for redox regulation and signalling. Thus, the role intracellular GSH/GSSG redox pair is an important determinant of intracellular redox status, influencing redox-regulated protein conformation, transcription etc. (see also 5.4.4.5).

CYSH/CYSS, in turn, largely determines extracellular redox state, which may control intracellular processes by redox-mediated signalling via intracellular ROS production (Imhoff et al. 2009). In addition, the intracellular redox states of GSH/GSSG and CYSH/CYSS are held at a considerably more reduced level than the ones in plasma, providing a reduced environment to intracellular processes (Jones et al. 2000).

In addition to CYSH/CYSS and GSH/GSSG, also homocyst(e)ine participates to the thiol status and has an important role especially in plasma, where its concentrations range from 5 to 12 μmol/l in healthy individuals, thus outnumbering the plasma GSH/GSSG pool. Less than 4% of plasma homocysteine is in its reduced, free, form (Ueland 1996) and the rest is in homocysteine disulphide form (homocystine), in mixed

disulphides with plasma cysteine (homocysteine-cysteine disulphide) or bound to protein sulphydryl groups, thus influencing extracellular redox status.

5.4.6.3. Thiol redox state as an indicator of oxidative stress or antioxidant reserves

In addition to its physiological role in redox regulation, thiol redox state can also be studied as an indicator of oxidative stress and antioxidant reserves. Changes in thiol redox state have been associated with many pathological states (Adams et al. 1983, Jones et al. 2002, Imhoff et al. 2009). In human plasma, the redox state of glutathione (GSH/GSSG) is about 60 mV more reduced that that of plasma cysteine (CYSS/CYSH), and they both are considerably more oxidised than the redox states in tissues or cultured cells. (Jones et al. 2000, Jones 2002). Thus, intracellular environment is held at a more reduced state than extracellular fluids.

The redox states of different thiol pools in human plasma (e.g. GSH/GSSG, CYSH/CYSS, cysteinylglycine/ its disulphides) are not equilibrated with each other. Instead, they are separately balanced so that the rate of thiol supply matches the rate of thiol oxidation in plasma, thus providing information about antioxidant/ oxidant status (Jones et al. 2000). The differences in redox states of plasma thiol pools suggest that the redox state of plasma cysteine/cystine pool represents oxidative events of plasma thiols – the first line of defence, oxidised before other thiols – whereas that of glutathione is indicative of plasma antioxidant capacity and the supply of antioxidants from tissues – the second line of defence (Jones et al. 2002).

When studying plasma thiol redox states, as is mainly done when studying human thiols *in vivo*, the main component is plasma CYSH/CYSS pool. The sources of changes in plasma GSH/GSSG redox state are much more complex and less interpretable (Blanco et al. 2007) and, in addition, more prone to artefactual results, as plasma GSH levels are low.

5.4.6.4. Glutathione and thiol status may reflect viability of cells and tissues

Glutathione and other thiols thus have many central roles in regulation, protection and even nutrition. Glutathione is more than an antioxidant or detoxifier; intracellular GSH, extracellular cysteine and protein thiol groups form a dynamic system known as the thiol status of the organism, which participates in the regulation of cellular redox environment and thus influences a broad spectrum of cellular functions and metabolic processes. All these roles taken together, glutathione and the thiol status may be seen as metabolic markers of well-being in cells or tissues with regard to their nutritional state, the accuracy of antioxidant defence, and cellular

viability including cell proliferation, differentiation and apoptosis (Pastore et al. 2003, Ballatori et al. 2009 b).

5.5. THIOLS IN HEALTH AND DISEASE

5.5.1. PHYSIOLOGIC VARIATION IN THIOL LEVELS AND THIOL REDOX STATE

Plasma and, possibly, tissue thiol levels and redox state vary according to several physiologic factors, including at least age, sex, diet and physical activity (**Table 4**). More oxidised plasma thiol redox state prevails during the newborn period (DiGiuseppe 2004) and in the elderly (Jones et al. 2002), and men have more reduced plasma thiols (Blanco et al. 2007). The redox state of plasma cysteine and/ or glutathione has a diurnal pattern (Blanco et al. 2007); plasma free reduced cysteine increases after meals, which reduces plasma cysteine redox state, and a similar phenomenon is seen with a 6-hour delay in plasma GSH concentrations and redox state. Dietary sulphur amino acid intake, also when adequate, influences plasma thiol redox states (Park et al. 2010, Jones et al. 2011), even if plasma thiol levels are tightly controlled by the liver (Stipanuk et al. 2006). In addition, other life style factors (e.g cigarette smoking, physical activity) may play a role (Moriarty et al. 2003, Banerjee et al. 2003).

5.5.2. FROM INBORN ERRORS OF GLUTATHIONE METABOLISM TO ACQUIRED STATES WITH LOW GLUTATHIONE LEVELS

The multiple roles of GSH are illustrated in the rare known conditions with inborn errors in GSH metabolism in humans (Meister and Anderson 1983, Meister 1991, Wu et al. 2004). In a defect in γ-GCS function (OMIM #266130), also known as 5-oxoprolinuria, γ-glutamylcysteine is produced in excess because of absent feedback inhibition from GSH, and this is further metabolised to 5-oxoproline, which accumulates leading to potentially lethal acidosis. The patients also have haemolysis and a neurodegenerative disease, probably due to absence of GSH antioxidant activity. All known inborn errors of GSH metabolism are associated to severe neurological problems. This clearly reflects the antioxidant, neuroprotective and, possibly, neuromodulative roles GSH plays in the central nervous system (Ballatori et al. 2009 b). In addition, patients with defects in GR, G6PDH, GCS and GS activity all present with haemolysis (Meister 1991), emphasising the priority of GSH in erythrocyte antioxidant defence (see 5.4.5.3).

Transgenic animals with deficiencies in GSH synthesis or function have been developed, essentially showing the same problems than patients with these defects (Halliwell 2007). In addition, GSH synthesis has been inhibited by specific agents in experimental animals. These studies show that the most important effect of extreme GSH deficiency is mitochondrial damage, and that newborn animals are more sensitive than older ones (Meister 1991).

In addition, multiple acquired situations with low GSH levels, mainly in plasma, have been reported, including malnourishment, critical illness, HIV infection, diabetes mellitus and Parkinson's disease (reviewed by Ballatori et al. 2009 b.). It is, however, difficult to define to which state GSH deficiency is inherent to the disease process itself, and which of the symptoms or metabolic problems may be related to GSH deficiency. A well-known and clinically relevant example of an acquired situation with GSH deficiency is paracetamol (acetaminophen) intoxication, where the electrophilic (oxidising) metabolite formed from acetaminophen is scavenged by GSH, leading to GSH depletion in the liver. This is followed by oxidative stress, mitochondrial dysfunction and, ultimately, liver cell necrosis and liver failure (James et al. 2003, Hinson et al. 2010).

Table 4. Physiologic variation in thiol levels and redox states.

Biologic or physiologic variable	**Influence to thiol status**	**Reference**
Age	1. Increasing age (after 50 y) associated with more oxidised plasma thiol redox state	1. Samiec et al. 1997; Jones et al. 2002; Blanco et al. 2007
	2. Newborn babies have more oxidised plasma thiols	2. DiGiusepppe et al. 2004
Sex	Plasma CYSH/CYSS more reduced in men	Blanco et al. 2007
Diet		
1. Protein intake	1. a) Decreased tissue GSH in experimental animals on low-protein diet	1. a) Taylor et al. 1992
	1. b) Decreased erythrocyte GSH and GSH synthesis rates during low (but adequate) protein intake	1. b) Jackson et al. 2004
2. Sulphur amino acid intake	2. Increased plasma CYSH and plasma CYSH/CYSS redox state more reduced with high sulphur amino acid intake	2. Park 2010
Day time	Diurnal variation in thiol redox according to meal time (more reduced plasma CYSH/CYSS after meals; followed by delayed reduction of plasma GSH/GSSG)	Blanco et al. 2007
Other habits/ life style factors		
1. Physical activity	1. Exercise associated with increased thiol levels; strenuous and prolonged exercise with thiol oxidation	1. Banerjee et al. 2003
2. Cigarette smoking	2. Smokers have oxidation of plasma CYSH/CYSS and GSH/GSSG	2. Moriarty et al. 2003

5.5.3. THIOLS IN METABOLIC DISEASES

Metabolic diseases often cause serious problems in the energy metabolism of cells and tissues; this may impair the function of thiol antioxidant systems, which are dependent on continuous energy supplies. Patients with metabolic diseases may also have poor nutritional status including low dietary protein intake, which potentially affects thiol status dependent on sulphur amino acid intake.

In addition to the possible impairment of thiol antioxidant systems, increased free radical production has been associated to many metabolic diseases including organic acidaemias, mitochondrial diseases, and homocystinuria. Increased free radical generation also seems to play a role in the pathogenesis of hypoglycaemic neuronal damage. The mechanisms involved are discussed in more detail in section 5.3. If free radical generation is simultaneously increased, any compromise in thiol metabolism would be detrimental.

5.5.3.1. Protein intake and nutrition influence thiol status and glutathione synthesis

Decreased tissue GSH concentrations have been measured in experimental animals fed with a low-protein diet (Taylor 1992), and similar results have been seen in protein-energy malnourished human subjects. Patients with anorexia nervosa had low plasma glutathione (Zenger 2004), and children with kwashiorkor had low erythrocyte GSH synthesis rates (Reid 2000, Badaloo et al. 2002). Thus, low GSH in protein-energy malnourishment apparently results from an inability to synthesize enough GSH and not only from an increase in GSH consumption; consequently, cysteine supplementation to protein-energy malnourished children also led to an increase in erythrocyte GSH synthesis (Badaloo et al. 2002).

It seems that even suboptimal, not insufficient, protein intake may decrease GSH synthesis in experimental animals (Stipanuk et al. 1992) and in humans. Healthy adults consuming the minimal amount of protein that is considered sufficient (0.75 g/kg/d for adults) had decreased erythrocyte GSH synthesis rates, preasumably following insufficient cysteine intake (Jackson et al. 2004). Interestingly, however, septic paediatric patients on limited nutritional support had decreased whole blood glutathione synthesis rates despite high plasma cysteine concentration and flux (Lyons et al. 2001). It seems that decreased GSH synthesis rates and/ or low GSH levels may occur in various forms of critical illness even in the absence of actual protein-energy malnourishment, presumably following a catabolic state that disfavours synthetic processes.

The findings about the possible deficiency in GSH precursors and/ or GSH synthetic capacity in various disease states and stress situations has led to reflections about whether the current recommendations for nutritional sulphur amino acid

intake should be revised (Tesseraud et al. 2008). However, excess cysteine must be avoided, as high levels are toxic (Stipanuk et al. 2006).

Additionally, it has been shown that nutrition (diet and fasting) not only influence thiol levels, but also thiol redox state. Cysteine and GSH redox states in human plasma, probably reflecting the situation in less accessible tissues, are more oxidised during fasting, becoming more reduced after meals (Blanco et al. 2007). Sulphur amino acid (methionine and cysteine) intake is shown to influence plasma thiol redox states with a more reducing state prevailing during high sulphur amino acid intake and vice versa (Jones et al. 2011, Park et al. 2010).

5.5.3.2. Decreased thiol antioxidant supplies in some inherited metabolic diseases

Organic acidaemias

Even if oxidative stress has often been suggested to play a role in the (neuro) pathogenesis of organic acidaemias (Wajner et al. 2004), little is known about the function of antioxidant systems in these diseases. Recently, it has been speculated that organic acids, such as MMA, may impair mitochondrial glutathione transport due to an inhibitory effect of dicarboxylic acids to the transporter (Morath et al. 2008), as it is known that organic acids inhibit the mitochondrial dicarboxylate carrier (Mirandola et al. 2008). Defects in mitochondrial GSH transport could have major consequences, as mitochondria are unable to synthesize GSH; their antioxidant defences are dependent on GSH transport by the dicarboxylate and the 2-oxoglutarate carriers (Lash 2006). It has also been shown that mitochondrial GSH status is critical for mitochondrial function an influences mitochondrial ROS production, which participates to regulation of cell viability and signalling (Fernandez-Checa et al. 1998; Lash 2009).

Moreover, more evidence form deficient antioxidant supplies in organic acidaemias comes from several *in vitro* studies that report a positive response to antioxidant administration following exposure to organic acids, expressed as improvement of cell survival (McLaughlin et al. 1998) or reduced neurological defects in experimental animals (Fighera et al. 1999, Pettenuzzo et al. 2002). There is also a case report about a positive response to antioxidant therapy in a patient with a severe metabolic crisis of methylmalonic acidaemia (Treacy et al. 1996), but patient studies are lacking.

Following the role of GSH as a detoxifier in conjugation reactions, it is interesting to speculate whether GSH could be depleted due to extensive conjugation reactions in organic acidaemias. Organic acids may accumulate to a thousandfold normal levels, and as wide array of chemical compounds are excreted as GSH conjugates. Under such exceptional metabolic circumstances GSH depletion due to a massively increased use in conjugation reactions could occur. However, it has not been studied

whether organic acids are included in the possible substrates for GST-catalysed reactions (Ballatori et al. 2009 a).

Hypoglycaemia

Cells need energy to produce GSH and maintain antioxidant capacity, and glucose is the preferred substrate for energy metabolism in most tissues. Glucose is also needed for NADPH production and NADPH, in turn, provides the reducing power for GR to regenerate GSH from GSSG. Thus it is reasonable to assume that antioxidant systems could be compromised during hypoglycaemia. There is, nevertheless, only limited information about the function of thiols and other antioxidant systems during hypoglycaemia, and results are partly contradictory.

According to some studies, glucose deprivation *in vitro* leads to a decrease in GSH (Li et al. 1998, Singh et al. 2004), and in experimental animals, plasma oxidised to reduced glutathione ratio (GSH/GSSG) decreases following insulin-induced hypoglycaemia (Jiang and Sato 1999). However, in an *in vitro* study on thiol levels in glucose-deprived erythrocytes (Coleman et al. 2007), total thiol levels did not change during glucose deprivation despite the presence of methaemoglobin as a sign of oxidative stress. Moreover, when the glucose-deprived cells were treated with the antioxidant lipoic acid, thiol levels actually decreased.

Studies, especially *in vivo,* about the function of antioxidant systems during hypoglycaemia are scarce. In two studies focusing on the effects of hyperinsulinaemia to oxidative stress markers in healthy human subjects, whole-blood GPx activity (and erythrocyte superoxide dismutase activity) decreased during insulin-induced hypoglycaemia (Koska et al. 1997, Koska et al. 2000). The authors do not discuss the significance of these findings and the possible association of hypoglycaemia to compromised antioxidant activity.

Mitochondrial diseases

ATP energy is needed for the maintenance and function of antioxidant systems, including GSH synthesis and transport to mitochondria, and impairment of energy metabolism may well influence antioxidant supplies. Accordingly, patients with defects in the function of the electron transport chain had decreased tissue GSH levels (Hargreaves et al. 2005). Moreover, poor antioxidant capacity has been shown to further compromise mitochondrial function (Bolanos et al. 1996, Hargreaves et al. 2005). Especially the redox status of thiols is critical for mitochondrial function (Lash 2006); mitochondrial GSH status regulates mitochondrial function and ROS production (Fernandez-Checa et al. 1998, Lash 2009).

However, the redox state and metabolism of thiols in diseases with compromised mitochondrial function have not been studied in detail, and the existing studies have led to conflicting results. In cultured human fibroblasts derived from patients with Complex I deficiency (Verkaart et al. 2007) no changes in thiol redox status were observed, whereas in patients with Friedreich's ataxia (Piemonte et al. 2001), blood glutathione redox status shifted towards increased oxidation, which was also observed in a yeast model of Friedreich's ataxia in addition to decreased intracellular glutathione levels (Auchère et al. 2008).

5.5.4. THIOL THERAPY: INCREASING THIOL LEVELS

As low GSH levels, both in plasma and in other tissues, are associated with a wide variety of disease states, it is a reasonable approach to try to increase blood and, ultimately, tissue GSH levels. However, GSH cannot be supplemented by itself, as it is a hydrophobic (unsoluble) molecule. Even if mechanisms for GSH uptake exist in some cells, they are not significant under physiologic conditions (Lash 2009, Ballatori et al. 2009 a); in addition, GSH is very short-lived (less than 3 minutes) in human plasma (Wendel and Cikryt 1980). Thus, attempts have been made to develop GSH derivatives with increased solubility or products enhancing GSH synthesis.

5.5.4.1. Glutathione esters and other glutathione derivatives

GSH ester prodrugs acting as soluble GSH transport vehicles include GSH monomethyl-, monoethyl-, diethyl- and isopropyl esters (Cacciatore et al. 2010). GSH esters bypass the ATP-requiring enzymatic steps required for GSH synthesis from its precursors, and are independent of the negative feedback that GSH exerts to GCS. GSH esters are also able to penetrate mitochondrial GSH pool.

GSH derivatives with modified cysteinyl moiety have also been developed. S-acetyl-GSH, S-phenylacetyl-GSH and L-cysteine-glutathione mixed disulphide are more stable and soluble than GSH and increase reduced intracellular sulphydryl groups, providing both GSH and cysteine to the cell (Cacciatore et al. 2010).

However, *in vivo* evidence is on GSH derivatives is scarce, not to mention clinical studies. As GSH has complex interorgan transport and homeostasis mechanisms, results from studies on GSH uptake by cultured cells are not directly adaptable to the whole human organism.

Table 5. Mechanisms underlying increased free radical production or compromised antioxidant function during several metabolic problems (reviewed in chapters 5.3.6. and 5.5.3.).

Studies with negative results are not represented in the table but are reviewed in the text. References are marked with a superscript number in the table and listed in numeric order under the table.

Disorder	Increase in ROS production; Possible mechanism favouring ROS production	Compromised thiol antioxidant function
Mitochondrial diseases	**"Leakage" of ROS** from defective ETC [1, 2]: increased • **hydroxyl radical** [3] • **superoxide** [4, 5] • **hydrogen peroxide** [5] production in cultured fibroblasts from patients with Complex I deficiency.	**Decreased muscle GSH** in patients with ETC defects [17] **Oxidation of blood GSH** in patients with Friedreich's ataxia [18] **Oxidation of GSH and decreased GSH** in a yeast model of Friedreich's ataxia [19]
Organic acidaemias	PA and MMA **inhibit mitochondrial energy production** [6, 7, 8, 9, 10]; mitochondrial dysfunction leads to ROS production as described above. PA and MMA **inhibit membrane Na+K+ATPase** [9, 11, 12] increasing free intracellular calcium and ROS production	MMA may impair **mitochondrial glutathione transport** [20] **Possible loss of GSH in conjugation** reactions following accumulation of organic acids (not studied).
Hypoglycaemia	Increased **hydrogen peroxide** [13] **and superoxide** [13, 14] production in brain tissue of experimental animals following insulin-induced hypoglycaemia. Release of **nitric oxide** in brain tissue of experimental animals following insulin-induced hypoglycaemia [15] Activation of **NADPH oxidase**, leading to **superoxide** production following glucose deprivation in cultured cortical neurons [14] Increased intracellular and mitochondrial **superoxide, hydrogen peroxide and peroxynitrite** in cultured retinal cells with glycolysis inhibition [16]	Glucose deprivation *in vitro* leads to a **decrease in GSH** [21, 22] Plasma **GSH/GSSG ratio decreases** in insulin-induced hypoglycaemia in experimental animals [23] **GSH depletion** in cultured retinal pericytes following glucose reduction [21] **Vitamin E prevented cellular injury** induced by glycolysis inhibition in cultured retinal cells [15]

[1] Wallace 1999; [2] Chen 2003; [3] Luo et al. 1997; [4] Pitkänen and Robinson 1996; [5] Wani et al. 2008; [6] Stumpf et al. 1980; [7] Nakai et al. 1991; [8] Dutra et al. 1993; [9] McLaughlin et al. 1998; [10] Okun et al. 2002; [11] Wyse et al. 1998; [12] Wyse et al. 2000; [13] McGowan et al. 2006; [14] Suh et al. 2007; [15] Suh et al. 2008; [16] Rego et al. 1999; [17] Hargreaves et al. 2005; [18] Piemonte et al. 2001; [19] Auchère et al. 2008; [20] Morath et al. 2008); [21] Li et al. 1998; [22] Singh et al. 2004; [23] Jiang and Sato 1999

5.5.4.2. N-Acetylcysteine and other cysteine prodrugs

Under most circumstances, cysteine is the limiting component of GSH synthesis and thus the most logical approach would be to supplement cysteine. This approach naturally requires that the ability of cells to synthesise GSH form its precursors is not affected. Glycine and glutamate, also needed for GSH synthesis, are generally found in abundance compared to cysteine, and are thus not efficient for promoting

GSH synthesis. Cysteine is unstable and and its therapeutic window is narrow (Taylor et al. 1992); it is easily oxidised to cystine, which is toxic due to low solubility in neutral pH. Thus, several cysteine prodrugs including L-2-Oxothialzolidine-4-carboxylate (OTC) (Taylor 1992) and N-Acetylcysteine (deVries 1993) have been developed. N-Acetylcysteine (NAC) is in clinical use, which is, however, limited, due to its suboptimal pharmacologic properties (Cacciatore et al. 2010).

5.5.4.3. Lipoic acid

α-lipoic acid (1,2-dithiolane-3-pentanoic acid, or thioctic acid) is a dithiol compound synthesised from octanoic acid in mitochondria, where it participates to energy metabolism by acting as a cofactor for α-ketoacid dehydrogenases. Beyond this role, lipoic acid seems to serve multiple purposes: it is a potent antioxidant, a detoxifier, and a modulator of inflammatory response. Lipoic acid has been used in clinical practice for treatment of diabetic retinopathy and neuropathy. Its safety in moderate doses has been demonstrated in several clinical trials, reviewed by Shay et al. 2009, although some questions over optimal dosage and safety in large doses remain.

As a thiol antioxidant, lipoic acid participates to maintenance and regulation of thiol redox status and thiol levels, and it is able to increase intracellular GSH levels (Bast 1988, Shay et al. 2009, Suh et al. 2004). Lipoic acid seems to increase cysteine availability by reducing cystine to cysteine, enhancing its uptake by cells; in addition, lipoic acid induces GSH synthesis by influencing redox-regulated signalling cascades and activating Nrf2-dependent transcription of antioxidant response element (ARE) –genes (Suh et al. 2004, Shay et al. 2009).

This far, several candidates for increasing intracellular thiols exist; lipoic acid is perhaps the most promising agent. Still, evidence from clinical studies is needed to evaluate the possibilities lipoic acid and other thol agents may offer for treatment – and whether increasing thiol levels truly offers therapeutic potential.

6. OBJECTIVES OF THE STUDY

This series of studies has two objectives, both dealing with biochemical changes in situations where metabolic perturbations occur. These changes are seen as metabolic markers of the underlying pathogenetic processes, offering new insights into disease mechanisms.

(1) **To study thiol metabolism in patients with different inborn errors of metabolism** (organic acidaemias, hypoglycaemic episodes, mitochondrial diseases, homocystinuria) and in cell culture models, where similar metabolic conditions are created.

Thiol homeostasis and thiol redox status, serving multiple metabolic and regulatory purposes, might be affected in inborn errors of metabolism following increased oxidative stress and nutritional factors. The changes in thiol status could have a role in the development of complications in these diseases. If confirmed, these changes in thiol metabolism would provide new understanding on the pathogenesis of different inborn errors of metabolism and even offer therapeutic potential.

(2) **To investigate the metabolic disturbances in patients with cow's milk allergy** (CMA) as a potential new diagnostic tool.

The composition and the metabolic activity of intestinal flora, which can be regarded as a tissue with substantial metabolic activity, are altered in patients with CMA or other food allergies. In addition, intestinal permeability to microbial products may be increased. This could lead to metabolic changes in the host. If measurable changes are present, these could be investigated for diagnostic purposes and as markers of metabolic effects of inappropriate nutrition.

7. MATERIALS AND METHODS

7.1. HUMAN SAMPLES

7.1.1. PATIENTS WITH INBORN ERRORS OF METABOLISM OR SUSPECTED HYPOGLYCAEMIA

17 children with suspected hypoglycaemic episodes, 10 children with a diagnosis of mitochondrial disease, 11 children with methylmalonic, propionic or isovaleric acidaemia and 6 children with homocystinuria attending the metabolic outpatient clinic in Great Ormond Street Hospital, London, UK, were enrolled in 1999-2000. The characteristics of the patients are summarised in **Table 6** and presented in more detail in the original publications I, II and III.

Among the patients with mitochondrial diseases, nine of the ten children had confirmed deficiencies of one or more of respiratory chain enzyme complexes I, II+III and IV, in skeletal muscle and/or fibroblasts. Four (40%) had a genetic diagnosis, including three with mitochondrial DNA mutations and one with a homozygous *SURF1* mutation.

The patients with organic acidaemias were supervised by a metabolic dietitian and received individually tailored treatment including carnitine, glycine (IVA only), metronidazole, vitamin supplements etc, and some had additional medication because of renal problems or epilepsy. None of them was severely protein restricted.

The body mass index standard deviation scores (BMI SDS) were calculated using the British 1990 growth reference (Cole 1995).

7.1.2. CONTROLS FOR PATIENTS WITH ORGANIC ACIDAEMIAS, MITOCHON DRIAL DISEASES AND HOMOCYSTINURIA

Reference data for comparison of values in patients with mitochondrial diseases, organic acidaemias and homocystinuria were derived from children referred to the same clinic for suspicion of hypoglycaemia caused by metabolic disease. These children were then found to be well, and no metabolic or any other disturbance could be identified after thorough clinical and laboratory investigation including a diagnostic fast (**Table 6.**).

Table 6. Characteristics of the patients with inborn errors of metabolism and their controls.

DISORDER	N	AGE (range, y)	SEX (M/F)
Mitochondrial disease	10	3-14	4/6
Organic acidaemia	11	1-16	8/3
isovaleric acidaemia	2	1.3-6.4	0/2
methylmalonic acidaemia	7	3.4-16	5/2
propionic acidaemia	2	5.7-12.2	2/0
Suspected hypoglycaemia	17	0.5-10.7	12/5
When fasted, plasma glucose below 3.0 mmol/l in	7	1.7-10.7	4/3
Homocystinuria	6	1-11	4/2
CONTROL	6	2.2-6	4/2

7.1.3. PATIENTS WITH SUSPECTED CMA

Patients in this study were a randomly selected subset (n=35) of a larger study cohort (n=230) that was studied between 1999 and 2002 in the Skin and Allergy Hospital in Helsinki University Central Hospital, Helsinki, Finland (Viljanen et al. 2005).

Children under 12 months of age with symptoms suggestive of CMA, atopic eczema being the obligatory symptom, and having received no probiotic treatment for 6 weeks before the study were enrolled.

7.1.4. ETHICAL ASPECTS

7.1.4.1. Patients with inborn errors of metabolism and suspected hypoglycaemia

In patients with mitochondrial diseases, organic acidaemias and homocystinuria, the blood samples for this study were taken under a routine follow-up visit at the same time than other blood samples for clinical purposes. For patients with suspected hypoglycaemic episodes, the samples were taken before and after a diagnostic fast with other blood samples included in the fasting protocol.

When appropriate, the children gave informed consent to participate to the study. When the child's age or condition prevented this, informed consent was obtained from the child's parents or guardians. The research ethics committee of GOSH/ Institute of Child Health, University of London, approved the study.

7.1.4.2. Patients with suspected CMA

The local ethics committee approved the study protocol and informed consent was obtained from one parent of each participating infant.

One parent of each infant gave informed consent to participate to the study. The urine samples from the patients with suspected CMA were taken under the follow-up visits needed for the exclusion or confirmation of CMA. The Ethics Committee of the Skin and Allergy Hospital of the Helsinki University Central Hospital approved the study protocol.

7.1.5. SPECIFIC STUDY PROTOCOLS

7.1.5.1. Fasting protocol for investigation of suspected hypoglycaemia

The maximum duration of the fast, ranging from 8 to 24 hours, was determined from the history and the age of the child. The fast was discontinuated before the planned time if the child developed clear neuroglycopaenic symptoms. An indwelling cannula was placed to collect the blood samples and to give intravenous glucose if necessary.

During the fast, blood glucose was measured hourly and the child's clinical condition was watched carefully to identify hypoglycaemia without delay.

A blood sample was taken both after a normal feeding interval (1st sample) and after the total duration of the fast (2nd sample).

7.1.5.2. Probiotic administration and CMA diagnosis

The study protocol is explained in detail in (Viljanen et al. 2005) and briefly summarised in **Figure 5**.

The infants with suspected CMA were first assessed for the severity of atopic eczema by the Severity Scoring of Atopic Dermatitis (SCORAD). Skin prick tests (SPT) for several relevant allergens including CM and serum specific IgE concentrations for cow's milk and wheat were examined, and a urine sample was taken (1st visit). Infants were then randomised by a computer-generated block randomisation in blocks of 6 infants to receive either *Lactobacillus rhamnosus GG* (LGG) or placebo in a double-blind trial for 4 weeks, after which they were examined again, and a new urine sample was taken (2nd visit).

Prior to the 1st visit, all the infants in both groups had been continuously exposed to cow's milk in some form (either directly from the infant's or indirectly from the mother's diet). Only after the 1st visit, cow's milk was eliminated from the infants' and their breast-feeding mothers' diets for eight weeks; an extensively hydrolysed whey formula was given instead. For those with

a favourable response to elimination diet, a double-blind placebo-controlled cow's milk challenge was then performed to confirm or exclude the diagnosis of milk allergy.

After confirmation or exclusion of CMA, urine samples of 16 infants with positive double-blind placebo-controlled food challenge (DBPCFC) and SPT to CM (CMA group) and 19 infants with a negative challenge and with no detectable allergies or sensitisations to any of the studied allergens (eczema only group) except for one egg-sensitized child with no egg introduced in the diet) were randomly selected for analysis. For the patients with CMA, urine samples both before and after LGG or placebo treatment were analyzed.

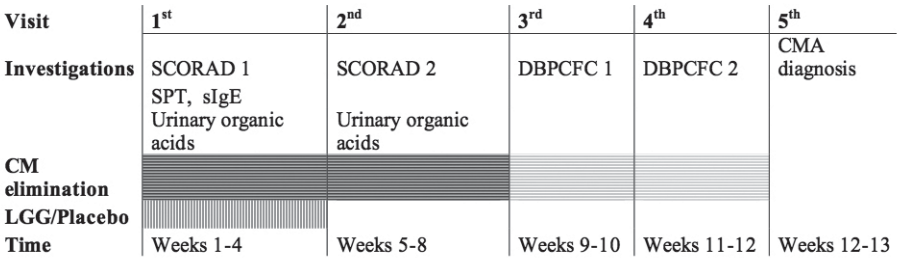


Figure 5. Study protocol (IV)

CM elimination started only after the first visit. Urinary organic acids were studied before and after LGG or placebo treatment in 35 infants. At the time of the first analysis, the children were exposed to CM, and at second analysis, CM had been eliminated and LGG or placebo administered.

The analyses were blinded to the patients' condition, as the diagnosis was made only after the DBPCFC several weeks later.

DBPCFC = double-blind placebo-controlled food challenge

7.1.6. BLOOD SAMPLES FROM PATIENTS WITH INBORN ERRORS OF METABOLISM AND THEIR CONTROLS

A blood sample was taken after informed consent during a routine follow-up visit to the clinic or, in patients with suspected hypoglycaemia, before and after the diagnostic fast. To control for the possible influence of fasting and diurnal variation to plasma thiols, all samples were taken after an overnight fast. The sample was kept on ice and centrifuged within 15 min to separate plasma from erythrocytes.

The erythrocyte pellet was frozen at −80°C, plasma was frozen as such and after protein precipitation with sulphosalicylic acid.

For analysis, erythrocytes were haemolysed with distilled and purified water.

7.1.7. URINARY ORGANIC ACID ANALYSIS IN PATIENTS WITH AND WITHOUT CMA

All urine during 5 hours was collected in an adhesive urine bag, cooled immediately to +4 °C, and stored at -20 °C. During the first 2 hours of the collection, infants were allowed nothing by mouth.

For gas chromatography/mass spectrometry analysis, urine samples were thawn. A special dip-stick (Dip ´N Dry, US BioTek, Seattle, WA, USA) was dipped in the urine and dried according to manufacturer's instructions. Creatinine was measured in addition to 37 stable easily identified stable organic acids (adipate, α-hydroxybutyrate, α-keto-β-methyvalerate, α-ketoglutarate, α-ketoisocaproate, α-ketoisovalerate, benzoate, β-hydroxybutyrate, citrate, cis-aconitate, ethylmalonate, fumarate, hippurate, homovanillate, hydroxymethylglutarate, isocitrate, kynurenate, lactate, malate, methylmalonate, orotate, para-hydroxyphenyllactate, pyroglutamate, pyruvate, quinolenate, suberate, succinate, tartarate, tricarbyllate, vanilmandelate, 2-hydroxyphenyllactate, 2-indoleacetate, 5-hydroxyindoleacetate). All acid concentrations were normalized to creatinine level. The assays were done blinded to the patient data.

7.2. CULTURED CELLS

7.2.1. HUMAN FIBROBLASTS EXPOSED TO ORGANIC ACIDS

Human fibroblasts from healthy volunteers were obtained with normal procedure and cultured in 37 °C and 5% CO2. The medium was RPMI 1640 with 10% foetal bovine serum and 2% streptomycin-penicillin. The medium was changed every two days. The cells were grown on 6-well plates to confluence and incubated with propionic acid (50 μM; 0.5 mM; 5 mM), methylmalonic acid (10 μM, 0.1 mM; 1mM) or isovaleric acid (10 μM; 0.1mM; 1mM) for 24 hours (2 wells for each incubation), after which a sample was taken of the media and cells were washed and harvested. The samples were stored in -80°C until analysis.

7.2.2. GLUCOSE-DEPRIVED HEPG2 CELLS (HUMAN HEPATOBLASTOMA CELLS)

The cells were grown on 6-well plates in DMEM, which had either normal glucose, half of normal glucose or no glucose for 28 h (12 wells for each), after which a sample was taken of the media and cells were washed and harvested. The samples were stored in -80 C until analyis.

7.2.3. GLUCOSE-DEPRIVED 293T CELLS (TRANSFORMED HUMAN EMBRYONIC KIDNEY CELLS)

The cells were grown, harvested and stored otherwise as HEPG2 cells, but samples were taken at 24 h and 48 for each glucose concentration (4 wells for each).

7.3. THIOL ANALYSIS

7.3.1. PLASMA THIOLS

The concentrations of glutathione and cysteine with (corresponding to total) and without (reduced thiols only) dithiotreitol (DTT) were measured by HPLC in the plasma samples with previously described methods (Ahola et al. 2004). In short, sulphydryls were derivatised with monobromobimane. A reversed-phase C-18 column was used to separate the adducts and quantitation was performed using a fluorescence detector.

7.3.2. ERYTHROCYTE THIOLS

In haemolysed erythrocytes, only total glutathione and cysteine were measured by the HPLC-based method described above, as haemolysis increases the oxidized fraction unpredictably. The glutathione and cysteine concentrations in haemolysed erythrocytes were normalised to the protein content of the samples.

7.3.3. THIOLS IN CULTURED CELLS

The concentrations of glutathione and cysteine with (corresponding to total) and without (reduced thiols only) dithiotreitol (DTT) were measured by HPLC in the cultured fibroblasts as previously described for plasma thiols (7.3.1).

7.4. ERYTHROCYTE ENZYME ACTIVITIES

GPx, GR, GST and G6PDH activities were measured in the haemolysed erythrocytes with a spectrophotometric assay as described earlier (Ahola et al.

2004). The assay detects the speed of formation of NADPH, which is measured spectrophotometrically using 340 nm as wavelength (the absorption maximum of NADPH).

Glucose 6-phosphate and NADPH were purchased from Boehringer Mannheim GmBH, NADP from Roche and all other chemicals from Sigma. Protein concentration was determined with the Bio-Rad kit.

All measurements were done in duplicate, and the mean value was calculated and normalised to erythrocyte protein content. In addition, also the absorbances of plain reagents and plain sample (without reagents) were controlled to check for possible iatrogenous enzyme activity.

The enzyme activity assays are explained in detail below.

7.4.1. GLUTATHIONE PEROXIDASE

The activity of GPx was determined in 0.10 M potassium phosphate buffer (pH 7.0) with 1.00 mM EDTA, 0.09 mM NADPH, 0.17 mM GSH, 10 mM tert-butyl-hydroperoxide and 23 IU GR.

7.4.2. GLUTATHIONE REDUCTASE

GR, the enzyme activity was measured in 0.10 M potassium phosphate buffer (pH 7.0) with 1.00 mM EDTA, 0.09 mM NADPH and 1.00 mM GSSG.

7.4.3. GLUCOSE 6-PHOSPHATE DEHYDROGENASE

The activity of G6PDH was determined in 0.10 M potassium phosphate buffer (pH 8.0) with 1.00 mM NADP, 1.00 mM glucose-6-phosphate and 0.50 mM dithiotreitol.

7.4.4. GLUTATHIONE TRANSFERASE

The assay was performed with 0.85 mM 1-chloro-2,4-dibenzene in 0.10 mM potassium phosphate buffer (pH 6.5) with 1.50 mM GSH.

7.5. OXIDATIVE DAMAGE TO ERYTHROCYTE LIPIDS AND PROTEINS

Oxidative damage to erythrocyte lipids and proteins was measured by determining the concentrations of a thiobarbituric acid –reactive species, malondialdehyde (MDA) and carbonylated proteins, respectively.

MDA concentration was measured in a thiobarbituric acid (TBA)–based spectrophotometric assay, described by Draper in 1993 and outlined below. Protein carbonyls were determined spectrophotometrically in the presence of 2,4-dinitrophenylhydrazine as published by Levine in 1994 and described below.

7.5.1. THIOBARBITURIC ACID –REACTIVE SUBSTANCES (TBA-RS)

For the measurement of TBA-RS, a reagent consisting of 0.67% thiobarbituric acid and acetic acid (1:1) was prepared. A mixture containing hemolysed red cells, water, the reagent and 1.22 M phosphoric acid (volumes 1:9:5:5, respectively) was incubated for 60 minutes in 95 C. After incubation, the absorbances of the samples were measured spectrophotometrically, using 532 nm as wavelength. To calculate the MDA levels from the measured absorbance, 1.54×10^5 x l/(M x cm) was used as MDA absorption coefficient.

7.5.2. PROTEIN CARBONYLS

To determine the amount of carbonylated proteins, 20% trichloroacetic acid (TCA) was added to the samples to precipitate the proteins. These were then let react with 10 mM 2,4-dinitrophenylhydrazine for 60 minutes (1 mg of protein and 500 µl of 2,4-DNPH). The reaction was stopped with 20% TCA and the sediment was washed three times with mixed ethanol and ethylacetate (1:1). 6M guanidine was added to the sediment and, after incubation for 15 minutes in 37 C, the absorbance of the supernatant was measured spectrophotometrically (wavelength 365 nm).

7.6. STATISTICAL ANALYSIS

7.6.1. COMPARISON OF PATIENTS WITH ORGANIC ACIDAEMIAS, MITOCHONDRIAL DISEASES AND HOMOCYSTINURIA TO CONTROLS

As the data was not normally distributed, statistical significance of measured parameters was tested by two-sided nonparametric Mann-Whitney U-test. Two-sided exact p was calculated with Statistica 7 software, and values < 0.05 were considered statistically significant.

7.6.2. ANALYSIS OF THIOL LEVELS BEFORE AND AFTER FASTING IN PATIENTS WITH SUSPECTED HYPOGLYCAEMIA

The concentrations of plasma thiols at the end of the fast (2nd sample) were compared to the concomitant plasma glucose value, and Pearson's correlation coefficient (R) was calculated.

The patients were divided in hypoglycaemic (plasma glucose below 3.0 mmol/l the end of the fast) and normoglycaemic (the rest) groups. The use of this particular value as a cut-off limit may be argued; this is discussed in detail in section 5.1.2.

The changes (expressed as the arithmetical difference) in erythrocyte thiol concentrations from the 1st sample to the 2nd sample were compared between the hypoglycaemic and normoglycaemic groups by unpaired t-test. A two-tailed probability (p) < 0.05 was considered statistically significant.

In addition, the statistical significance of the relative proportions of changes to opposite directions in erythrocyte thiol levels in these two patient groups was tested with Statistica 7 software, and p values < 0.05 were considered statistically significant.

7.6.3. COMPARISON OF URINARY ORGANIC ACIDS IN PATIENTS WITH AND WITHOUT CMA AND BEFORE AND AFTER LGG TREATMENT

Normal distribution of data was assured by logarithmic conversion of the measured values, and the mean, standard deviation and median were calculated for each subgroup. To evaluate the effects of LGG treatment, the difference in urinary organic acid concentrations following administration of either placebo or LGG was calculated.

Statistical significance between the patients with and without CMA and before and after treatment was tested by two-tailed unpaired t-test and $p<0.05$ was considered statistically significant.

8. RESULTS

8.1. THIOLS IN PATIENTS WITH INBORN ERRORS OF METABOLISM

Basic information (including diagnosis, age, sex, height, weight and clinical condition at time of blood sampling) was available from nearly all patients and all controls. The height and weight of three patients with hypoglycaemia were missing. One patient with mitochondrial disease was unable to stand, and the height had not been measured.

All plasma samples from participating patients were included for thiol analysis except for one patient with isovaleric acidaemia, whose plasma as well as erythrocyte samples were unavailable at the time of analysis. This patient, having no data to analyse, was excluded from the study. One patient with homocystinuria was studied twice, having an acute respiratory infection during the first visit. Only the blood sample from the second visit was used for data analysis, as also all other patients were studied during routine control visits without any acute problems.

Due to a small number of patients and controls, the variation in all measured thiols was wide in patients and in controls. The data was not normally distributed.

All values represent mean ± standard deviation (SD) unless otherwise stated. p values are derived from several different statistical tests according to the data and hypotheses that are tested, as is explained in 7.6.

8.1.1. PLASMA THIOLS AND THEIR REDOX STATE

Both free (reduced) and total glutathione and cysteine in plasma were measured. **Total cysteine** comprises free (i.e. reduced) cysteine, cystine (i.e. the oxidised disulphide form), and protein-bound (i.e. oxidised) cysteine. **Total glutathione** comprises free (i.e. reduced) glutathione, glutathione disulphide (i.e. the oxidised disulphide form), and protein-bound (i.e. oxidised) glutathione.

The redox state is here assessed as CYSH/CYSS ratio, GSH/GSSG ratio or by comparing plasma thiols before and after plasma protein precipitation. True redox potentials have not been calculated.

The analysis of plasma thiols before and after protein precipitation allows estimation of the amount of protein-bound glutathione or cysteine, which is always oxidised. If a greater fraction of plasma total glutathione or cysteine is protein-bound, then the plasma glutathione or cysteine pool is more oxidised.

Patients with organic acidaemias, hypoglycaemia, mitochondrial diseases and homocystinuria had altered levels of plasma thiols as compared to controls. Their

plasma thiol redox status was indicative of oxidative stress even in the absence of acute critical illness.

8.1.1.1. Plasma cysteine

Cysteine is the most important non-protein thiol in plasma. In this series of studies, all studied inherited metabolic diseases were associated with lower levels of plasma free cysteine and, hence, a more oxidised plasma cysteine redox state.

Plasma cysteine in patients with mitochondrial diseases
Patients with mitochondrial diseases had significantly lower levels of reduced cysteine in plasma than controls (12.7 ± 2.7 μmol/l vs. 19.5 ± 5.6 μmol/l, p = 0.008; **Figure 6**), whereas their total plasma cysteine levels did not differ. Thus, the reduced to total cysteine ratio in plasma of patients with mitochondrial diseases was significantly decreased (0.063 ± 0.026 vs. 0.097 ± 0.021, p = 0.023; II).

Plasma cysteine in patients with organic acidaemias
Total plasma cysteine levels were similar or slightly higher in patients with organic acidaemias as compared to controls (245.7 ± 32.9 μmol/l vs. 218.1 ± 26.6 μmol/l; **Figure 6**), but the concentration of free (reduced) cysteine, CYSH, was lower in patients than in controls (III). Consequently, the ratio of reduced to oxidised cysteine (CYSH/CYSS) was lower in the plasma of the patients with organic acidaemias.

Plasma cysteine in hypoglycaemic patients
Following fasting, the levels of reduced cysteine in untreated plasma correlated significantly to the blood glucose level ($R=0.63$, $p<0.02$), suggesting that a decrease in blood glucose is associated with a decrease in reduced cysteine in plasma (I).

Plasma glucose was not correlated to levels of oxidised cysteine (CYSS) or to levels of total cysteine in plasma.

Plasma cysteine in patients with homocystinuria (unpublished data)
Total plasma cysteine levels were significantly lower both in untreated and in acid-treated plasma of patients with homocystinuria as compared to controls (170.4 ± 34.8 vs. 218.1 ± 26.6 μmol/l, p = 0.026; and 102.1 ± 28.5 vs. 181.8 ± 82.1 μmol/l, p = 0.041, respectively; **Figure 6**). The levels of free cysteine were lower in patients but did not differ significantly between patients and controls, making the CYSH/CYSS ratios similar (0.099 ± 0.03 vs. 0.097 ± 0.02). However, patients with homocystinuria tended to have more protein-bound (oxidised) plasma cysteine than controls, but the variation was wide (the ratio of protein-bound cysteine to total plasma cysteine was 0.40 ± 0.10 vs. 0.14 ± 0.47 in patients and controls,

respectively). This suggests, albeit indirectly, that the patients' plasma cysteine pool was more oxidised in a similar way than that of patients with organic acidaemias, mitochondrial diseases and hypoglycaemia.

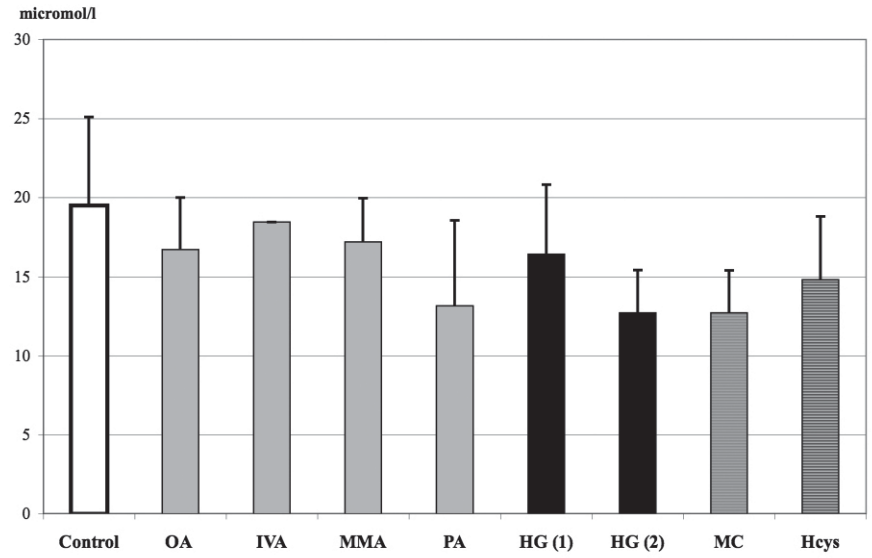


Figure 6. Plasma free = reduced cysteine (CYSH; Mean + SD) in patients with
OA = (any) organic acidaemia
IVA = isovaleric acidaemia
MMA = methylmalonic acidaemia
PA = propionic acidaemia
HG (1) = hypoglycaemia, 1st sample
HG (2) = hypoglycaemia, 2nd sample (during hypoglycaemia)
MC = mitochondrial diseases
HCys = homocystinuria
Plasma levels of total cysteine did not differ markedly between patients and controls except for the patients with homocystinuria with lower plasma total cysteine levels.

8.1.1.2. Plasma glutathione

Plasma free (reduced) glutathione concentrations are very low, in the micromolar range. Consequently, in this series of studies, plasma free GSH concentrations were often below the detection limit of the analysis. This decreases the power of the study to find statistically significant changes in plasma free glutathione levels or plasma glutathione redox status.

To assess the redox state of plasma GSH/GSSG pair when GSH levels were too low to be measured, we compared plasma total GSH before and after precipitation of plasma proteins as explained in 8.1.1.

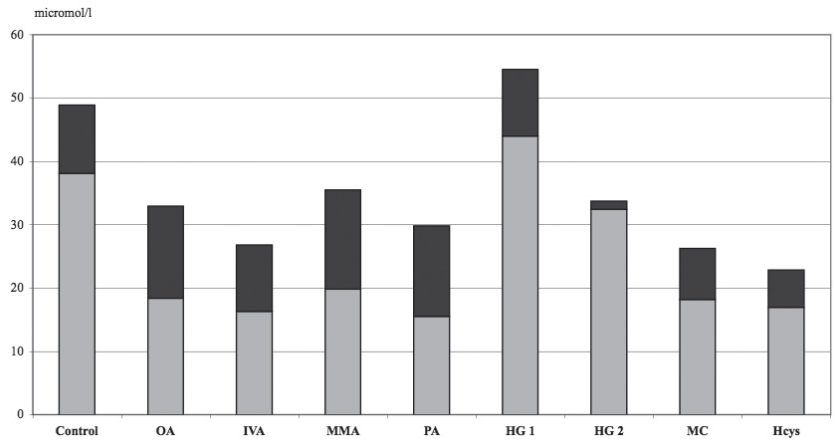


Figure 7. Total plasma glutathione in patients with
OA = (any) organic acidaemia
IVA = isovaleric acidaemia
MMA = methylmalonic acidaemia
PA = propionic acidaemia
HG 1 = hypoglycaemia, 1st sample
HG 2 = hypoglycaemia, 2nd sample (during hypoglycaemia)
MC = mitochondrial diseases
Hcys = homocystinuria
Black bars represent the protein-bound fraction of total plasma glutathione.
Shaded bars represent the unbound fraction of total plasma glutathione.

Plasma glutathione in patients with mitochondrial diseases

Plasma concentrations of total glutathione (including oxidised glutathione i.e. glutathione disulphide, GSSG, and reduced glutathione, GSH) were lower in patients with mitochondrial diseases as compared to controls (26.3 μmol/l vs. 48.9 ± 25.7 μmol/l; p= 0.031; II), and a greater fraction of the patients' plasma glutathione was protein-bound (oxidised, as explained in 8.1.1) (**Figure 7**).

These two indirect approaches suggest that patients with mitochondrial diseases also had lower plasma levels of reduced glutathione. Indeed, the measured concentrations of reduced glutathione in plasma were lower in patients with mitochondrial diseases, below the detection limit in nearly all patients, whereas most control patients had clearly measurable plasma free glutathione concentrations. This suggests that had the analysis been more sensitive to very low GSH concentrations, patients with mitochondrial would probably have had significantly lower plasma free GSH levels. However, in the presence of several results below the detection limit, no statistical analysis was possible.

Plasma glutathione in patients with organic acidaemias

There were no statistically significant differences in plasma glutathione levels between patients with organic acidaemias and controls. However, patients with organic acidaemias had a lower plasma concentration of total glutathione than the

control group (32.9 ± 6.9 μmol/l vs. 48.9 ± 25.7 μmol/l; **Figure 7**; III); patients with isovaleric or propionic acidaemia had clearly lower plasma total GSH levels than those with methylmalonic acidaemia, but the small number of patients with each specific organic acidaemia makes statistically relevant subgroup analyses impossible.

The difference between healthy controls and patients increased when plasma samples were treated with acid before analysis. Thus, in patients with organic acidaemias, a greater fraction of plasma glutathione was oxidised as mixed disulphides with proteins, as explained in 8.1.1. Again, this indirectly suggests that they had lower plasma levels of reduced glutathione, difficult to measure directly.

The levels of free GSH in plasma of patients with organic acidaemias were low, mostly below the detection limit of the analysis, not allowing reliable comparison between patients and controls.

Plasma glutathione in hypoglycaemic patients

Levels of free GSH were low also in patients with hypoglycaemia, and no statistically significant differences between patients and controls or correlation of GSH to blood glucose could be observed (R = 0.39, N.S; I). Levels of oxidised glutathione (GSSG) were not correlated to plasma glucose (R = 0.45, N.S.), and also total glutathione concentrations remained unaffected by plasma glucose (R = 0.44, N.S.).

Plasma glutathione in patients with homocystinuria (unpublished data)

Patients with homocystinuria had significantly lower plasma levels of total glutathione as compared to controls (22.8 ± 4.0 vs. 48.9 ± 25.7, p = 0.015). (**Figure 7)**

Levels of free GSH in plasma were two low to allow statistical comparison, or assessment of plasma glutathione redox state.

8.1.2. THIOLS IN HAEMOLYSED ERYTHROCYTES

In haemolysed erythrocytes, only total thiols (i.e. oxidised + reduced thiols) were measured.

In patients with organic acidaemias and mitochondrial diseases, erythrocyte total thiols were similar to the levels in healthy controls (**Table 7**).

In patients who became hypoglycaemic after fasting, erythrocyte cysteine levels were lower before the fast (0.017 ± 0.022 μmol/l vs. 0.039 ± 0.022 μmol/l); the fast induced an increase in total cysteine in the haemolysed erythrocytes of hypoglycaemic patients (0.017 ± 0.022 μmol/l to 0.045 ± 0.023 μmol/l) (**Figure 8; Table 7**). This was not seen in the control patients ($p < 0.05$ for the change in erythrocyte cysteine as compared to the control group). An increase in erythrocyte during fasting was seen in only one control patient vs. 5/7 hypoglycaemic patients ($p < 0.05$ for the comparison of the relative proportions of changes to opposite

directions). The increase in erythrocyte cysteine was not reflected in erythrocyte glutathione levels, as there was no corresponding change in the patients' erythrocyte total glutathione (7.04 ± 1.50 μmol/l to 7.05 ± 1.32 μmol/l).

Table 7. Erythrocyte total non-protein thiols (I, II, III)

	Erythrocyte total cysteine μmol/g protein	**Erythrocyte total glutathione** μmol/g protein
Control	0.039 ± 0.022	6.7 ± 0.56
Mitochondrial diseases	0.041 ± 0.025	6.4 ± 1.1
Organic acidaemias	0.038 ± 0.030	6.6 ± 1.5
Hypoglycaemia (1)	0.017 ± 0.022	7.0 ± 1.5
Hypoglycaemia (2)	0.045 ± 0.023	7.1 ± 1.3

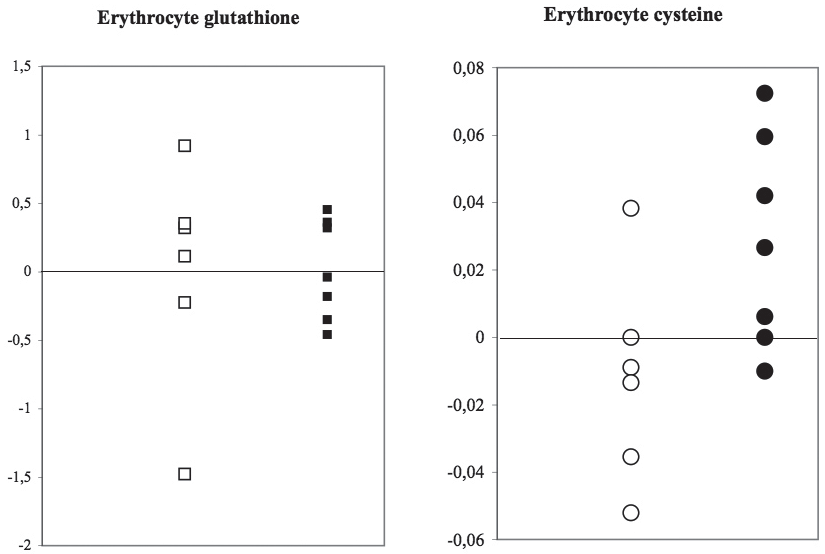


Figure 8. Difference in erythrocyte total cysteine (left panel) and erythrocyte total glutathione (right panel) before and after the diagnostic fast.

Values below 0 indicate a decrease in erythrocyte cysteine during the fast in a particular subject.

Open marks: controls
Black marks: patients

8.2. THIOLS IN CULTURED CELLS

8.2.1. THIOLS IN CULTURED HUMAN FIBROBLASTS EXPOSED TO ORGANIC ACIDS (III)

Both reduced and total glutathione concentrations in fibroblasts decreased after 24 h exposure to increasing concentrations of isovaleric and propionic acid. When exposed to methylmalonic acid, the concentrations of both reduced and total glutathione decreased first, but were then increased following exposure to a higher concentration of methylmalonic acid (Figure 9). The ratio of reduced glutathione to oxidised glutathione decreased following exposure to methylmalonic and propionic acid.

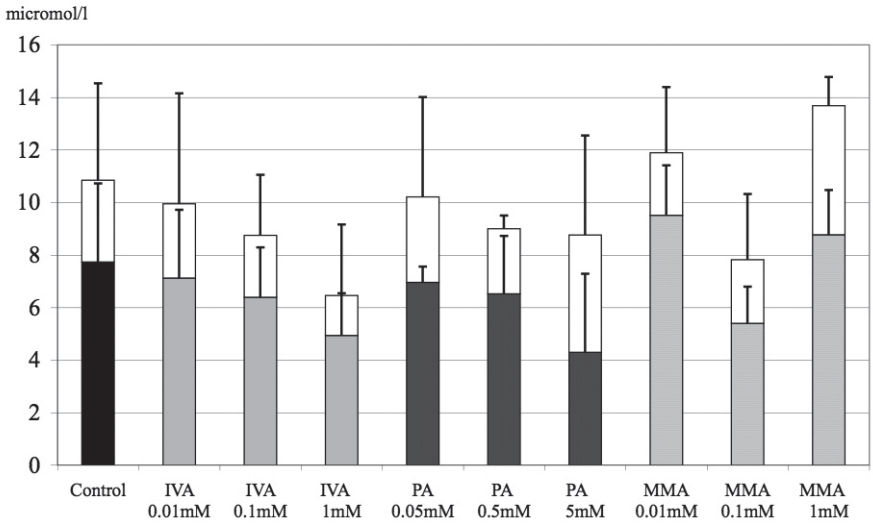


Figure 9. Reduced (black or shaded bars) and oxidised (open bars) **glutathione** in cultured human fibroblasts exposed to organic acids. Mean + SD is represented (SDs for both total and reduced glutathione).

Similar results – increased thiol oxidation, but not consistent findings with all three acids – were obtained with cysteine levels in fibroblasts exposed to organic acids (**Figure 10**). The concentration of reduced cysteine was lower in fibroblasts exposed to IVA, MMA and PA as compared to the control level. In addition, the ratio of reduced to oxidised cysteine was lower than the control level in fibroblasts exposed to the highest levels of the three organic acids.

In the cell culture media, the concentrations of glutathione or cysteine did not change markedly following exposure to organic acids, indicating that no major uptake or release of glutathione or cysteine occurred.

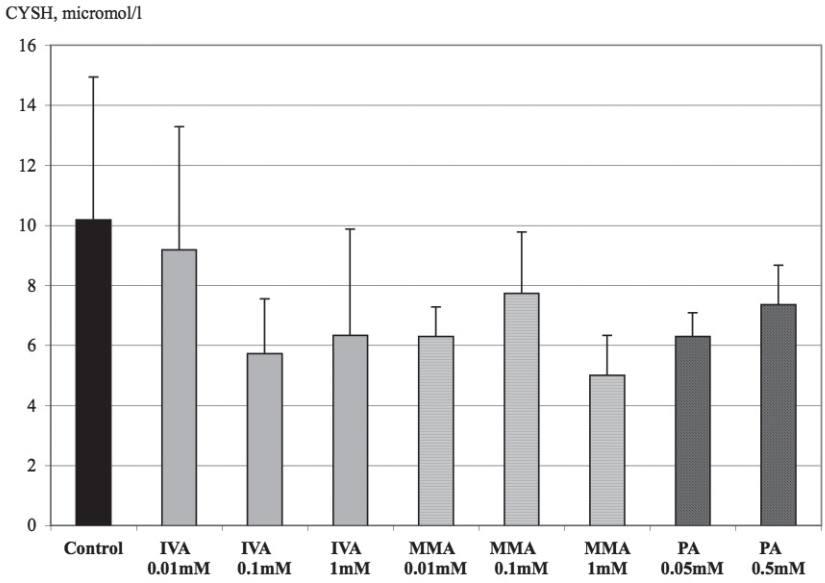


Figure 10. Reduced cysteine (CYSH) in cultured human fibroblasts exposed to organic acids. Mean + SD is represented.

Levels of reduced cysteine after exposure to 5 mM PA were under the detection limit and are not shown.

8.2.2. THIOLS IN CULTURED HEPG2 AND 293T CELLS EXPOSED TO LOW GLUCOSE OR GLUCOSE DEPRIVATION

In cultured HEPG2 cells, 28-hour glucose deprivation lead to 41% loss of free GSH and 23% loss of total glutathione, as compared between cells grown in normal glucose content and those completely deprived of glucose. The values of cells grown in half of normal glucose were intermediate between these two (**Figure 11**). CYSH and total cysteine values did not change markedly during glucose deprivation (unpublished data).

On the contrary, in cultured 293T cells, 24-hour glucose deprivation lead to an increase in both CYSH (+150%) and GSH levels (+35%); also total cysteine and glutathione levels increased (**Figure 11**). However, when glucose deprivation was prolonged to 48 h, both CYSH and GSH and corresponding total thiol levels decreased clearly (unpublished data)

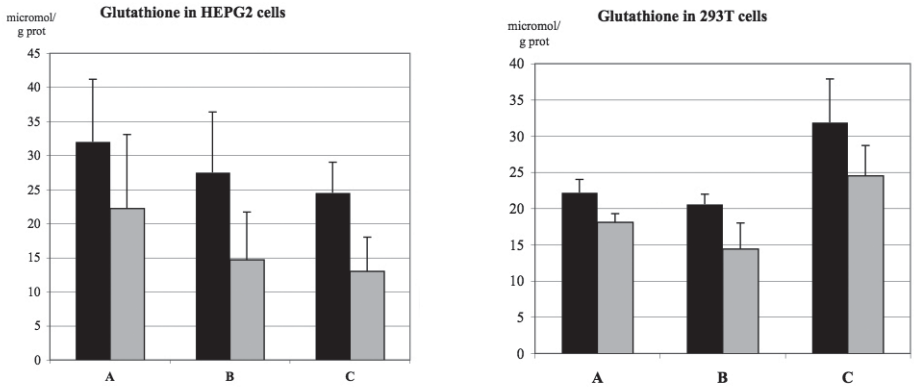


Figure 11. Total glutathione (black bars) and **free GSH** (shaded bars) in **HEPG2** cells (left panel) and **293T** cells (right panel) cultured in **(A)** DMEM with normal glucose **(B)** DMEM with half of normal glucose **(C)** DMEM with no glucose. Mean + SD is represented.

8.3. ACTIVITIES OF ENZYMES RELATED TO GLUTATHIONE ANTIOXIDANT AND DETOXIFYING FUNCTION IN HAEMOLYSED ERYTHROCYTES OF PATIENTS WITH INBORN ERRORS OF METABOLISM

There were no statistically significant differences in GPx, GR, G6PDH or GST activities in patients with mitochondrial diseases or organic acidaemias as compared to the controls (Table 8; II, III).

The activities of GPx, GR, G6PDH or GST did not change significantly during fasting, did not correlate to blood glucose levels and there were no significant differences between hypoglycaemic and normoglycaemic patients (Table 8; I).

Table 8. Erythrocyte enzyme activities (I, II, III)

	Erythrocyte GPx activity 10^3 IU/ mg prot.	**GR activity** 10^4 IU/ mg prot.	**G6PDH activity** 10^4 IU/ mg prot	**GST activity** 10^4 IU/ mg prot
Control	2.1 ± 0.47	4.6 ± 2.2	3.9 ± 1.1	6.0 ± 5.7
Mitochondrial diseases	2.0 ± 0.69	5.5 ± 1.9	4.3 ± 0.72	5.3 ± 5.0
Organic acidaemias	1.87 ± 0.75	3.8 ± .4	4.4 ± 2.7	5.2 ± 4.1
Hypoglycaemia, 1st sample (when normoglycaemic)	1.84 ± 0.80	5.5 ± 1.3	3.0 ± 0.93	4.1 ± 2.2
Hypoglycaemia, 2nd sample (during hypoglycaemia)	1.92 ± 0.62	4.8 ± 0.98	4.2 ± 0.81	4.9 ± 2.9

8.4. OXIDATIVE DAMAGE TO ERYTHROCYTE PROTEINS AND LIPIDS IN PATIENTS WITH INBORN ERRORS OF METABOLISM

No statistically significant differences in erythrocyte MDA or protein carbonyl levels were noticed in patients with any of the studied inborn errors of metabolism (**Table 9**; I, II).

However, variations were wide for MDA levels in all patient groups as well as controls; with these results and with a small number of patients and controls implying a risk for type II error, the reliability of the whole analysis must be questioned. Due to technical problems, results for the MDA levels for two of the children in the control group were missing, which decreases the statistical power to detect differences between patients and controls.

These results (the absence of increased erythrocyte lipid peroxidation despite evidence about a more oxidised plasma redox state) thus need to be interpreted with much caution.

Table 9. Oxidative damage to erythrocyte lipids and proteins (I, II)
: data from two controls missing

	Erythrocyte MDA µmol/l	**Protein carbonyls** mmol/l
Control	1.5 ± 1.5 #	0.045 ± 0.011
Mitochondrial diseases	1.8 ± 1.4	0.047 ± 0.011
Organic acidaemias	1.2 ± 1.2	0.049 ± 0.024
Hypoglycaemia, 1st sample (when normoglycaemic)	0.5 ± 0.5	0.041 ± 0.0064
Hypoglycaemia, 2nd sample (during hypoglycaemia)	2.0 ± 1.7	0.044 ± 0.0075

8.5. URINARY ORGANIC ACID PROFILE IN PATIENTS WITH COW'S MILK ALLERGY (IV)

Urinary organic acids were analysed from all the 35 participants. 16 of them had confirmed CMA, and their urinary organic acid profile was studied again after placebo or LGG treatment. This second urinary sample was not available for analysis for two of the CMA patients. This did not affect the pre-treatment results, where all the 35 samples were available, and did not cause a type I error in the post-treatment results, where no clearly significant results were seen.

Missing 2 out of 16 samples may have caused a type II error in the analysis of urinary organic acids after LGG or placebo treatment.

The levels of urinary organic acids as such were not normally distributed in this small study population. This problem was solved by logarithmic conversion of the measured values, and t test could be used to test statistical significances.

8.5.1. DIFFERENCES IN THE BASELINE URINARY EXCRETION OF ORGANIC ACIDS IN PATIENTS WITH CMA COMPARED TO PATIENTS WITH ONLY ATOPIC ECZEMA

Significant differences between CMA and eczema only -patients were noticed in the urinary concentrations of β-hydroxybutyrate ($p<0.001$; IV); adipate and isocitrate ($p<0.01$ for both; IV); homovanillate, suberate, tartarate, 3-indoleacetate and 5-hydroxyindoleacetate ($p<0.05$ for all; IV).

CMA patients had always a higher concentration than eczema only -patients and the concentrations did not correlate with the SCORAD values (**Table 10**).

8.5.2. EFFECTS OF PROBIOTIC (LGG) TREATMENT TO URINARY ORGANIC ACID PROFILE

After treatment with LGG, statistically significant changes between the treatment group and the placebo group were noticed only in the levels of kynurenate and 3-indoleacetate ($p<0.05$ for both; IV). In the concentrations of organic acids that were different between CMA and eczema only -patients, a trend towards the eczema only -level was seen; the urinary levels of these organic acids decreased during LGG administration in CMA patients (**Table 10**).

Table 10. (IV). Levels of urinary metabolic end products with significant differences between CMA and eczema only –patients before (1.) or after treatment with LGG (2.).

The data are expressed in µg/mg creatinine after logarithmic conversion.
1. Statistical difference between ezcema only and CMA groups. * = p<0.05; ** = p<0.01; *** = p<0.001
2. Statistical difference after LGG vs. Placebo treatment. # = p<0.05.

Study phase	**1. Before LGG treatment**		**2. After 4 weeks**	
Patient group	**Only eczema**	**CMA**	**CMA + LGG**	**CMA + Placebo**
Urinary metabolite	mean ± SD	mean ± SD	mean ± SD	mean ± SD
beta-hydroxybutyrate	0.51 ± 0.21	0.8 ± 0.22***	0.70 ± 0.32	0.63 ± 0.28
adipate	0.64 ± 0.4	1.00 ± 0.26**	0.83 ± 0.22	0.89 ± 0.48
isocitrate	1.8 ± 0.11	1.93 ± 0.11**	1.86 ± 0.1	1.98 ± 0.12
homovanillate	0.82 ± 0.19	0.93 ± 0.08*	0.85 ± 0.06	0.92 ± 0.13
kynurenate	0.67 ± 0.18	0.66 ± 0.15	0.71 ± 0.08	0.69 ± 0.11 #
suberate	0.39 ± 0.32	0.61 ± 0.18*	0.47 ± 0.22	0.63 ± 0.22
tartarate	0.38 ± 0.21	0.64 ± 0.35*	0.26 ± 0.13	0.98 ± 0.2
3-indoleacetate	1.06 ± 0.3	1.27 ± 0.27*	1.17 ± 0.29	1.47 ± 0.23 #
5-hydroxyindoleacetate	0.58 ± 0.31	0.78 ± 0.21*	0.76 ± 0.15	0.77 ± 0.41

9. DISCUSSION

9.1. THIOL STATUS IN INBORN ERRORS OF METABOLISM

Inborn errors of metabolism such as organic acidaemias, mitochondrial diseases and disorders leading to hypoglycaemia all present with problems in energy production and nutrient metabolism. This series of studies has shown that these disorders are associated with changes in plasma thiol levels and redox states. These changes are suggestive of increased thiol oxidation and, possibly, decreased thiol antioxidant (GSH) supplies.

9.1.1. ROLE AND RELEVANCE OF PLASMA THIOLS

In plasma, cysteine is the most abundant non-protein thiol. With levels exceeding a hundred-fold those of plasma glutathione, plasma cysteine is also more easily and reliably studied. Accordingly, the most significant changes we noticed in plasma thiols were in the plasma cysteine pool. Patients with mitochondrial diseases (II) and organic acidaemias (III) had evidence of increased plasma cysteine pool oxidation and lower plasma reduced cysteine levels. Reduced cysteine levels were also decreased in patients who became hypoglycaemic during fasting (I) and in patients with homocystinuria, who also had more cysteine bound to plasma proteins and, thus, a more oxidised plasma cysteine pool (unpublished data).

It is possible that plasma GSH was subjected to more subtle changes, and that similar differences would have been noticed in plasma GSH pool, had the number of patients and controls been greater. Indeed, several of the results suggest that. Patients with organic acidaemias (III) and mitochondrial diseases (II) mostly had plasma free GSH levels below the detection limit of the analysis, whereas most of the control patients had measurable concentrations. In organic acidaemias (III) and mitochondrial diseases (II) as well as in hypoglycaemic patients (I), a greater fraction of plasma total glutathione was bound to protein, which gives indirect evidence about oxidation of plasma GSH pool. In addition, in all of these patients and also in patients with homocystinuria, plasma total glutathione levels were lower than in controls; whether this is due to increased glutathione consumption or decreased glutathione synthesis – or both – cannot be stated, as GSH synthesis rates and fluxes were not studied.

Plasma thiols represent the net sum of a complex interorgan homeostasis of different thiol pools. Some authors suggest that plasma thiol levels are correlated to intracellular thiol levels in other tissues, including the liver (Adams et al. 1983,

Jones 2002, Pastore et al. 2003), and represent a long-term view of thiol metabolism in the body (Taylor et al. 1992). This is not an unjustifiable view, and it certainly is a tempting one, as obtaining plasma samples for research is more feasible than obtaining liver or lung biopsies.

Previous studies (Jones et al. 2002) suggest that the redox states of plasma thiol pools can be studied separately to gain understanding about the extent of oxidative events or the possible impairment of antioxidant defences. Plasma cysteine/cystine pool seems to represent oxidative events of plasma thiols – the first line of defence, oxidised before other thiols – whereas that of glutathione would be indicative of plasma antioxidant capacity and the supply of antioxidants from tissues – the second line of defence. Thus, even if thiol levels in other tissues may not always be reflected in plasma thiol levels, the redox state of plasma thiols could represent thiol redox states in other tissues.

It seems reasonable to state that plasma thiol levels probably reflect thiol concentrations in other tissues only to some extent. Instead, rather than extrapolating plasma thiol levels to unstudied tissues, studying plasma thiols is relevant for gaining information about the redox environment cells are subjected to. If changes in plasma thiols are noticed, it may not be possible to state the origin of these changes with certainty, but changes in extracellular redox state will have major metabolic consequences. Changes in extracellular redox environment, manifested in an increased CYSH/CYSS oxidation such as we noticed in most studied patients (II, III), have been shown to be associated with inflammatory response, apoptosis signalling, and many different disease states; they have far-reaching effects to cell cycle gene expression and cell proliferation (Park et al. 2009, Jones et al. 2011).

9.1.2. UNDERSTANDING THIOL STATUS BEYOND PLASMA THIOL LEVELS; INTRACELLULAR THIOLS

In order to understand thiol status, studying intracellular thiol levels is mandatory. Obtaining tissue samples from paediatric patients, however, is problematic. In this series of studies (I, II, III), intracellular thiols are represented by erythrocyte thiols in all patient groups. In order to create intracellular conditions similar to the metabolic disturbances encountered in organic acidaemias and hypoglycaemia, we also studied intracellular thiols in human fibroblasts exposed to organic acids and in HEPG2 and 293T cells deprived of glucose.

We noticed a significant increase in erythrocyte cysteine during hypoglycaemia (I). In order to study the effects of hypoglycaemia to thiol metabolism, erythrocytes are an optimal choice in the sense that they are dependent on glucose and glycolysis for their energy production. On the other hand, due to their glucose dependency, they may be less sensitive to low blood glucose concentrations. Erythrocytes are able

to transport glucose efficiently from rather low plasma concentrations, as the Km for the red cell glucose transporter, GLUT 1, is 1-2 mmol/l (Thorens et al. 1990). It is thus interesting that according to our results, blood glucose concentrations clearly exceeding that (2-3 mmol/l) had effects on erythrocyte thiols.

Erythrocytes have high GSH synthesis rates, but they lack γGT activity (Srivastava 1976) needed for GSH degradation for uptake and re-synthesis inside cells. Instead, erythrocytes transport the constituent amino acids cysteine, glycine and glutamate (in the form of glutamine) by specific sodium-dependent transporters (Raftos et al. 2010). The increase in erythrocyte cysteine cannot be explained by changes in cysteine availability, as plasma free cysteine levels during hypoglycaemia were low. Even if cysteine is glucogenic, cysteine is not targeted for energy metabolism in erythrocytes, as gluconeogenesis from amino acids mainly takes place in the liver and, possibly, in the kidneys and intestine during extended fasting (Mutel et al. 2011). Instead, the increase in erythrocyte free cysteine during low blood glucose may be seen as an antioxidant response aiming at increasing intracellular GSH synthesis in erythrocytes. As erythrocyte total glutathione levels remained unaffected by blood glucose, increased GSH synthesis did not take place during hypoglycaemia, possibly following impaired synthetic capacity and lack of ATP energy.

In cultured HEPG2 cells, 28-hour glucose deprivation lead to loss of free and total glutathione, whereas CYSH and total cysteine values did not change markedly during glucose deprivation (unpublished data). Thus, GSH levels in HEPG2 cells, which are an in vitro model of human hepatocytes, decreased either following deficient capacity to synthesise GSH, or following increased GSH export due to an increase in oxidative stress in other tissues.

On the contrary, in cultured 293T cells originating from human embryonic kidney cells, 24 h glucose deprivation lead to an increase in both CYSH and GSH levels; also total cysteine ad glutathione levels increased (unpublished data). However, when glucose deprivation was prolonged to 48 h, both CYSH and GSH and corresponding total thiol levels decreased clearly. Renal tubular cells have high γGT activity and are thus able to take GSH from the extracellular space for breakdown and intracellular resynthesis of GSH. It seems that glucose deprivation induced GSH synthesis from its constituents, but that after prolonged glucose deprivation, the cells' synthetic capacities became compromised.

Tumour cell lines (such as HEPG2) have an altered thiol metabolism as compared to healthy cells in a living organism; in addition, separate cell culture models can hardly mimic the complex interorgan metabolism of thiols. Still, our results with glucose-deprived 293T and HEPG2 cells fit quite well into current knowledge on the roles of different cells and tissues in thiol metabolism. During oxidative and metabolic stress, as occurs in hypoglycaemia, liver cells supply GSH for the use of other cells and tissues with high γGT activity, such as the kidney.

9.1.3. THE ROLE OF SOME DIETARY AND METABOLIC FACTORS IN THE ALTERED THIOL STATUS IN INBORN ERRORS OF METABOLISM

9.1.3.1. Protein-restricted diet may lead to changes in thiol status in organic acidaemias

We hypothesised that dietary protein restriction, even if the diet is balanced to meet specific amino acid requirements, may affect thiol status in certain metabolic disorders such as organic acidaemias. According to previous studies, dietary sulphur amino acid intake influences plasma free cysteine levels, with low intake leading to low plasma free cysteine concentrations and thus a more oxidised CYSH/CYSS redox state (Jones et al. 2011). According to the same study, these changes were not directly reflected in plasma GSH levels even if dietary sulphur amino acid intake (cysteine availability) mainly controls the rate of GSH synthesis. Blood GSH synthesis rates (but not necessary GSH levels) diminish when sulphur amino acid intake is inadequate (Lyons et al. 2000, Lyons et al. 2001), and even suboptimal – not insufficient - protein intake may decrease GSH synthesis (Stipanuk et al. 1992, Jackson et al. 2004).

Our results with patients with organic acidaemias (III) – low plasma free cysteine, more oxidised plasma cysteine pool, and decreased plasma total glutathione – are in line with these findings. Thus, the protein-restricted diet, even if matching nutritional recommendations, may be one explanation to the changes in thiol status of patients with organic acidaemias.

9.1.3.2 Influence of other dietary factors to thiol status: diurnal variation in plasma thiols

Plasma thiols show some diurnal and meal-associated variation. In one study with healthy adults (Blanco et al. 2007), plasma cysteine (CYSH) levels increased after meals, and plasma CYSH/CYSS redox state became more reduced towards the evening. Interestingly, this was followed by similar changes in plasma GSH and GSH/GSSG with a 6-hour delay. We measured plasma thiols simultaneously, and thus the possible delayed effects of the changes we noticed in plasma CYSH/CYSS status would not have been detected.

As for the diurnal variation in plasma thiols, all the samples in our study (I, II, III) were taken in the morning after an overnight fast; it is not likely that the patients would have had different diurnal patterns from the controls. However, patients with metabolic diseases and especially those with organic acidaemias and hypoglycaemic episodes have very special diets and eating patterns, sometimes also including frequent meals due to intolerance to fast. One could speculate about this

having an influence on the meal-associated variation in plasma thiols even if sulphur amino acid intake is not affected. However, our results – low free cysteine levels and redox states in patients with organic acidaemias and mitochondrial diseases – do not directly support this hypothesis.

9.1.3.3. Poor nutritional status and need for protein may underlie changes in plasma thiols

There was a positive correlation between blood glucose and free cysteine (I); low blood glucose was associated to low free cysteine levels. Cysteine is a glucogenic amino acid; it is possible that in the patients with lowest blood glucose, blood cysteine decreased following cysteine uptake in the liver for gluconeogenesis. Alternatively, it could be hypothesised that those with an ability to liberate amino acids, including cysteine, to be then used by the liver for glucose production did not become hypoglycaemic. Thus, nutritional status and protein intake might not have been adequate in the patients with low cysteine. This explanation is, however, unlikely, as total cysteine levels did not differ between patients and controls.

Patients with mitochondrial diseases (II) and organic acidaemias (III) also had lower plasma free cysteine levels. In both diseases, lack of substrates for (protein) energy metabolism may occur (in mitochondrial diseases following failure of efficient energy production and in organic acidaemias, following low-protein diet). Patients with mitochondrial diseases (II) and organic acidaemias also had lower total glutathione levels (III). This may reflect poor nutrition and need for more protein. Either GSH synthesis in the liver was impaired due to lack of protein, or a decrease in plasma GSH may be due to the amino acid transporter role of GSH (Meister and tate 1976). Tissues with high γGT activity may be responsible for an increased GSH degradation and uptake of constituent amino acids for protein synthesis.

9.1.3.4. Metabolic blockage in homocystinuria may explain decreased cysteine and glutathione levels

In homocystinuria due to CBS deficiency, the metabolic pathway generating cysteine from methionine is blocked (Mudd et al. 2001); homocysteine is an intermediate in the transsulphuration pathway (**Figure 2**), which regenerates cysteine from methionine. Thus, patients with homocystinuria due to CBS deficiency or defect have absent or decreased ability to make cysteine, which is normally not an essential amino acid. This could lead to lack of cysteine, and accordingly, we noticed significantly decreased plasma cysteine levels. Logically, this may also lead to decreased GSH synthesis, as cysteine is the rate-limiting component of GSH

synthesis; indeed, decreased total plasma glutathione was observed in patients with homocystinuria.

Thus, patients with homocystinuria not only have altered plasma thiols following hyperhomocysteinaemia, where abnormally high levels of homocysteine disrupt other plasma thiol pools by thiol-disulphide reactions (Di Giuseppe et al. 2004); the metabolic blockage in CBS deficiency may also lead to secondary changes in thiol levels following defects in non-protein thiol synthesis.

9.1.4. PHYSIOLOGIC VARIATION IN PLASMA THIOLS

Due to the small number of patients and controls, it was not possible to control for the possible confounding factors that arise from physiologic variation in thiol levels related to age, sex, diet, physical activity, diurnal variation etc., overviewed in 5.5.1. and in **Table 4**. Possible effects of the diet on thiols were part of the study hypothesis, and it was not meaningful to control for those. All samples were taken in the morning after an overnight fast, so the possible confounding effects of fasting and daytime were controlled to a sufficient degree.

Our patients and controls were not matched for age; age is known to influence plasma thiols (Jones et al. 2002) at least in older age groups (middle age and after). Age-related variation in thiol levels in healthy children has not been studied thoroughly. In one study (Di Giuseppe et al. 2004), plasma reduced and oxidised thiols (plasma cysteine, glutathione, homocysteine and cysteinylglycine and corresponding disulphides) remained fairly constant after the newborn period throughout the childhood and puberty. Age-related physiologic variation (a decrease in plasma thiol redox status) was observed only when comparing newborns to other subjects or after 50 years of age. Our patients and controls were all between 1 and 16 years old; thus, even if the evidence is not exhaustive, it seems that our results on plasma thiols should not be biased by the different age distribution in the patient and control groups.

For future studies, it could be feasible to choose matched controls (at least matched for age and sex), as this would reduce the number of controls needed for enhancing statistical power. Variables possibly affecting thiol levels (age, sex, physical activity etc.) could be better controlled for. In addition, a pre-study power analysis would enhance the statistical validity of results; even if the number of patients was limited for obvious reasons, a power analysis would probably have led to increasing the number of controls in this series of studies.

9.2. ALTERED THIOL STATUS AS AN INDICATOR OF OXIDATIVE STRESS IN INBORN ERRORS OF METABOLISM

GSH has multiple different roles, but its main purpose is related to its antioxidant function. However, due to complex interorgan and intracellular compartmentalisation, glutathione levels are not straightforward markers of oxidative stress or antioxidant reserves.

In this series of studies (I, II, III), to address these questions, glutathione antioxidant function was studied from several points of view. First, both GSH and its precursor cysteine levels were studied intracellularly and extracellularly; second, GSH antioxidant function was investigated by measuring several GSH-dependent antioxidant enzyme activities. Finally, to gain understanding about the ultimate consequences of a possible increase in oxidative stress, oxidative damage to tissue proteins and lipid was studied.

The results of this series of studies support the idea of oxidative stress, here reflected in a change in plasma thiol status, playing a role in the disease process of organic acidaemias, hypoglycaemic episodes and mitochondrial diseases. Whether this is due to inadequate thiol antioxidant production, impaired function or increased consumption, cannot be fully understood with our results, as we did not find direct evidence of differences in antioxidant function (expressed by thiol antioxidant enzyme activities) or oxidative damage.

9.2.1. CHANGES IN PLASMA THIOL STATUS WITHOUT ANTIOXIDANT RESPONSE OR OXIDATIVE DAMAGE IN ERYTHROCYTES

We found evidence of changes in plasma thiol status, suggestive of an increase in oxidative stress, in several metabolic diseases. Still, there were no significant changes in erythrocyte antioxidant enzyme activities, nor was there evidence of an increase in oxidative damage to erythrocytes. The absence of a significant change in erythrocyte GSH-dependent antioxidant enzyme levels despite changes in plasma thiol status may suggest that the patients with mitochondrial diseases, hypoglycaemia and mitochondrial diseases were unable to produce an appropriate antioxidant response following increased thiol oxidation and compromised function of antioxidant systems.

The samples were taken under routine control visits, when the patients were not acutely ill or metabolically decompensated. Thus, oxidative stress, reflected in a change in plasma thiol status, seems to be present at all phases in these metabolic disturbances. This supports the role of oxidative stress in the pathophysiology of metabolic disorders; it not only occurs at a late stage when overwhelming metabolic stress, impairing all control mechanisms and leading to tissue damage, is present, but also throughout the disease.

As oxidative stress is a dynamic state between two players – antioxidant defences and free radical generation – it is not straightforward to interpret the results of antioxidant enzyme activities during oxidative stress. An increase in antioxidant enzyme activity may be seen as a stress response to an increase in free radical generation, or a measure preventing oxidative damage in healthy tissues. A decrease in antioxidant enzyme activity may reflect deficient antioxidant capacities, or a metabolic state with low ROS production and abundant antioxidant supplies. Indeed, in previous studies on antioxidant enzymes in metabolic disorders, the results about the association these enzymes to otherwise detected oxidative stress are contradictory. During hypoglycaemia, GPx activity has been reported to decrease (Koska et al. 2000), to remain unchanged (Patockova et al. 2003), or to increase (Singh et al. 2004). Similarly, during compromised mitochondrial function, GPx activity has been shown to increase (Auchère et al. 2008), decrease (Wani et al. 2008) or be equal to controls (Tozzi et al 2002).

Our results on enzyme activities and oxidative damage need, however, to be interpreted with caution. The number of patients and controls was small and the variations wide, possibly leading to absence of a statistically significant difference. In addition, our results (no increase in MDA an protein carbonyls in erythrocytes of patients with metabolic diseases) do not exclude the possibility of oxidative damage occurring in other tissues than erythocytes in response to the oxidation of plasma thiols. However, previous studies have shown that a change in extracellular redox status is not necessarily reflected to intracellular redox state (Imhoff et al. 2007), thus making intracellular oxidative damage unlikely. We used a TBA-RS –based assay to measure MDA levels as a marker of lipid peroxidation; the assay, however, has low ability to detect some products of oxidative damage to lipids, including oxidation products of arachidonic acid (De Zwart et al 1999).

In order to better understand the intracellular effects of altered plasma thiol status, complementary methods would be needed. These could include expression of oxidative stress –induced transcription factors and more exhaustive methods to study oxidative damage, including isoprostane analysis to detect the products of arachidonic acid oxidation (DeZwart et al 1999), and a method such as the 8OHdG assay to screen for oxidatively damaged nucleic acids (Halliwell 2007).

9.2.2. ROLE OF ERYTHROCYTES IN STUDYING THIOL ANTIOXIDANT SYSTEMS

Erythrocytes, although rich in antioxidant systems, have no nucleus and are thus very special cells. Due to the absence of protein synthesis in erythrocytes, they cannot be studied to gain understanding about possible changes in transcription activity of antioxidant enzymes, or enzymes affecting thiol metabolism. However,

erythrocytes are continuously exposed to high oxygen concentrations due to their role as oxygen carriers. Moreover, they are prone to ROS formation by the Fenton reaction due to the presence of high levels of transition metals (iron in the haem) (Halliwell 1991; Kurata et al. 1993).

Erythrocytes have deficient capacity to respond to oxidative stress due to the inability to respond by a change in transcription activity or synthesis of antioxidant enzymes. Thus, in many other respects, erythrocytes are relevant cells for studying oxidative damage, and also some forms of antioxidant responses, including thiol levels ant thiol antioxidant enzyme activity. Erythrocytes are fairly easily available for analysis even in a paediatric study setting, and they have thus often been used previously, facilitating comparison of results.

9.3. DETERMINATION OF THIOLS IN BIOLOGIC SAMPLES IS TECHNICALLY DEMANDING; LIMITATIONS OF THE STUDY

9.3.1. LOSS OF THIOL GROUPS AFTER SAMPLING

Measuring intra- and extracellular thiol levels and their redox state poses several technical problems due to the short half-time of the species and their predisposition to oxidation-reduction reactions (Jones et al. 1998; Newton and Fahey 1995). Intracellular thiol (mainly GSH) levels and especially their redox state may be strongly influenced by thiol auto-oxidation during sample procession, e.g. during haemolysis of erythrocytes for analysis. In addition, intracellular thiol-metabolising enzymes (GPx, GR etc.) may affect thiol redox states after sampling (Hwang et al. 1995). As intracellular GSH/GSSG pool is maintained at a very reduced state, even a small increase in GSSG due to sample auto-oxidation or post-sampling enzymatic activity would have a significant influence to the measured glutathione redox state. Intracellular GSH is also compartmentalised to cytosolic, endoplasmic and mitochondrial pools, and their participation to the measured intracellular thiol levels may be unpredictable (Hwang et al. 1995).

We only measured intracellular total thiols in patient samples, which minimises the previously described methodological problems. However, total thiols only represent the net sum of thiol oxidation-reduction reactions and also thiol (GSSG) efflux, and the interpretation of these is not straightforward. For instance, the increase in intracellular total cysteine in hypoglycaemic patients may result from increased cysteine uptake or decreased cysteine use for GSH synthesis. Any decrease in intracellular total GSH may be due to an increase in GSSG efflux, or decrease in GSH synthesis. On the other hand, if GSH use in antioxidant reactions increased,

this would remain unnoticed; if no significant GSSG export occurs, total GSH levels would remain unchanged, as was possibly the case in our studies.

Measurement of plasma (or extracellular) thiols is prone to similar artefacts following suboptimal sample collection and sample processing techniques. Especially measurement of plasma GSH levels is demanding, as the values are in the micromolar range. GSH in plasma is also very short-lived with a half-time of a few minutes at room temperature (Wendel and Cikryt 1980), and plasma γGT activity may contribute to plasma GSH degradation under some special circumstances (Jones et al. 1998). Most importantly, erythrocyte GSH levels are 500 times above plasma levels; thus, even 0.1% haemolysis (Jones et al. 1998) during blood sampling or before plasma separation may increase plasma GSH levels substantially.

When taking blood samples from children, the risk for unintentional haemolysis and artefactual thiol oxidation is even greater than in adults due to small veins, small needles used for venipuncture and to the need for using a tourniquet. Interestingly, in a previous study (Di Giuseppe et al. 2004), newborn subjects had increased plasma thiol and especially GSH oxidation when compared to older children or adults; the authors do not discuss the possible influence of technically difficult blood sampling leading to artefactual loss of blood GSH in the newborn period. These problems can hardly be avoided in an out-patient setting where arterial blood sampling is not possible. It would, however, be possible to include a marker for haemolysis (such as plasma haemoglobin) in plasma thiol analyses.

9.3.2. LACK OF REFERENCE VALUES – NEED FOR A CONTROL GROUP AND ADEQUATE NUMBER OF PATIENTS AND CONTROLS

Plasma GSH levels from different laboratories are not necessarily comparable. For instance, GSH levels in human plasma vary over 10-fold in the literature, depending on the analytical procedure used (Jones et al. 1998). It is not clear which analytical procedure is the most reliable (Meister and Anderson 1983), although monobromobimane-based thiol group alkylation followed by HPLC analysis, as was used in this series of studies, has been shown to be useful and reliable (Meister and Anderson 1983, Newton and Fahey 1995). Even using similar monobromobimane -based HPLC methods, reported plasma GSH values vary from under 1 μmol/l to 3-6 μmol/l (Jones et al. 1998).

Thus, it may not be possible to compare our absolute results with previous ones; that is also why the use of a control group, sampled and analysed identically with the patients, was mandatory. It would certainly have been ideal to include more children to the control group. Having a small number of controls implies a risk for type II error; especially in a study with a limited number of patients, a greater number

of controls would improve statistical validity of results. Obtaining relevant control samples is a difficult issue in clinical studies, and it is even more problematic, for obvious ethical reasons, when paediatric patients and controls are concerned. In this study, the small number of patients and controls is, to some extent, compensated by the fact that the controls were well chosen. They lived in the same area, where of similar age, and were thoroughly studied and found to be healthy.

The number of patients was small, which is inevitable as the studied diseases are very rare; this leads to a risk for both type I and II errors. In addition, for the same obvious reason, it was not possible to make a pre-study power calculation to estimate the number of patients and controls needed to reach satisfying statistical power. As we studied thiol metabolism from several points of view, we did not have a single hypothesis to test; this makes the study prone to type I errors due to multiple comparisons between patients and controls. However, as results with different metabolic diseases affecting energy and nutrient metabolism were very much alike – increased plasma thiol oxidation and decreased plasma glutathione levels – the results of multiple comparisons gain additional validity. Moreover, the objective of the study was to find new ideas for pathogenetic factors, but not directly to find new means of treatment. Thus, the risk of performing a type I error is ethically less problematic than type II error, which would lead to omitting true, possibly important, differences between patients and controls.

More patients (and controls) could have been recruited only by including multiple specialised centres from different countries, which could hardly have been justified when dealing with this kind of preliminary study hypothesis. Now that some metabolic diseases clearly are associated to changes in thiol metabolism, it would be of interest to study more patients and also GSH levels in tissues or cultured cells from patients.

9.4. ROLE OF THIOLS IN THE PATHOGENESIS OF COMPLICATIONS IN INBORN ERRORS OF METABOLISM

9.4.1. THIOLS, NEURODEGENERATIVE DISEASES AND NEUROLOGICAL SYMPTOMS IN INBORN ERRORS OF METABOLISM

Many inborn errors of metabolism present with neurological symptoms, and also complications of hypoglycaemia are due to neuronal damage. Some patients with inborn errors of metabolism, e.g. those with organic acidaemias, may also have structural brain abnormalities (Wajner et al. 2004), suggesting a disease mechanism profoundly influencing neuronal differentiation, function and survival.

Glutathione has a critical role in the central nervous system; besides being an antioxidant, it has been shown to have other neuroprotective and neuromodulative roles (Janáky et al. 1999, Oja et al. 2000, Ballatori et al. 2009 b). The clinical (or therapeutic) significance of these findings linking GSH to neuronal survival and central nervous system function is not clear, but the association is interesting. Inborn errors of GSH metabolism all present with central nervous system involvement, and low GSH have been reported in various neurodegenerative disorders (Ballatori et al. 2009 b), Sabens et al. 2011.) – including, as has been reviewed in this thesis, several inborn errors of metabolism. It seems that alterations in sulphydryl homeostasis leading to changes in redox signaling and regulation influence the pathogenesis of neurodegenerative diseases (Sabens et al. 2011).

The possible neuromodulative roles of GSH form an interesting, yet hypothetical, link between the observed changes in thiol metabolism and the poorly understood neuropathology in many inherited metabolic disturbances, including organic acidaemias, mitochondrial diseases and hypoglycaemic neuronal damage. To further evaluate this hypothesis, thiols in cells of neuronal origin should be studied.

9.4.2. ALTERED THIOLS AND ATHEROSCLEROSIS IN HOMOCYSTINURIA

Patients with homocystinuria often present with cardiovascular and thromboembolic complications (Clarke 1991), and elevated levels of homocysteine are associated to an increased risk of cardiovascular complications also in the general population (Homocysteine studies collaboration 2002).

The exact disease-causing mechanisms of elevated levels of homocysteine are unknown, but oxidative stress due to the pro-oxidant properties of homocysteine in blood has been suggested as an explanation (Welch and Loscalzo 1998). Homocysteine, when present at high levels, participates in thiol redox status and is able to form mixed disulphides with other plasma non-protein thiols and protein thiol groups (DiGiuseppe et al. 2004). It thereby significantly influences extracellular redox status and redox regulation. Accordingly, we found that patients with homocystinuria had clear changes in their plasma thiols with lower total glutathione and total cysteine levels. Even if we could not show a significant change in their plasma thiol redox state, there was indirect evidence of increased plasma cysteine oxidation (unpublished data).

Thus, homocystinuria is associated to altered plasma thiol levels and an altered plasma thiol redox state due to the ability of homocysteine to participate to thiol-disulphide reactions and disrupt extracellular thiol status. This forms an interesting link between the poorly understood pro-oxidant nature of homocysteine, oxidative stress and atherosclerosis in homocystinuria.

9.5. ALTERED THIOLS IN METABOLIC DISEASES; POSSIBILITIES FOR THIOL TREATMENT?

This thesis, focusing on thiols and their metabolism, did not concentrate on antioxidants, nor did the studies include antioxidant treatment. As we noticed changes in thiol status suggesting an increase in oxidative stress and a relative GSH deficiency, it is, still, tempting to speculate on the therapeutic implications these findings could have. Thiol status can be affected by adequate nutritional sulphur amino acid intake and, failing that, by thiol antioxidant supplementation.

As reviewed in 5.5.4, GSH as such cannot be used as a therapeutic agent. Despite intensive research, knowledge on the optimal product to improve GSH levels is still incomplete. Among agents that are targeted to increase cellular GSH levels, lipoic acid (1,2-dithiolane-3-pentanoic acid, or thioctic acid) is a promising one. It has been shown to increase intracellular thiol levels, and as a thiol antioxidant, it is able to truly participate to the complex mechanisms involved in the regulation of thiol redox status and redox signalling. In addition, compared to GSH or cysteine prodrugs, it better studied *in vivo* and even used clinically (Shay et al. 2009).

In fact, in most clinical situations, despite a well-justified scientific basis and clinical evidence of oxidative stress, antioxidant trials have failed; even with intensive research, antioxidant supplementation still remains a question of debate, and according to a meta-analysis, they may even be harmful (Bjelakovic et al. 2007). Even in disorders like mitochondrial diseases where excessive free radical production very clearly plays a role, evidence does not support the use of antioxidants (Chinnery et al. 2006).

It is probable that part of the problem is due to fact that knowledge on the mechanisms of action of specific antioxidants and, particularly, the interplay between different antioxidant systems is incomplete (Bjelakovic et al. 2007). Many antioxidant systems act in collaboration with each other, and the effects of depletion – or supplementation – of a single antioxidant are not predictable. In addition, most trials have used combinations of antioxidants without a clearly defined target antioxidant system. As many antioxidants may also have pro-oxidant effects, the optimal dosage of a particular antioxidant should have been determined before clinical trials. Due to this dual antioxidant/pro-oxidant character, it is also possible that the effects of an antioxidant in diseased cells or tissues will differ from those in healthy cells often used in study settings (Coleman et al. 2007).

Administering antioxidants such as α-linoleic acid (vitamin E) or ascorbic acid (vitamin C), as has been done in many clinical trials (Bjelakovic et al. 2008), may be too many steps upstream in the cascade initiating the disease process. From this point of view, understanding the role of thiols is relevant, notably following the ubiquitous roles thiols play in cellular survival and regulation. When a change in thiol status is noticed, it is possible to target interventions to thiol metabolism

– not just to administer any antioxidant in the hope of reducing oxidative stress arising from unknown sources.

Obviously, more research and understanding on the role of thiols in metabolic disturbances is needed before considering any therapeutic approaches. In addition, *in vivo* studies on thiol supplementation are lacking (with, perhaps, the exception of lipoic acid), even if numerous promising products have been developed.

9.6. CMA AND ALTERED URINARY METABOLIC END PRODUCTS

The urinary organic acid profile was altered in children with CMA as a sign of metabolic changes in the intestine due to either inappropriate nutrition and/ or differences in intestinal microbiota and its metabolic activity. The differences we noticed in urinary organic acid excretion in a clinically relevant diagnostic setting– infants with atopic eczema, but with or without CMA – may offer interesting new potential for CMA diagnosis.

9.6.1. A MODEL FOR FOOD INTOLERANCE? THE ROLE OF URINARY β-HYDROXYBUTYRATE AS A MARKER OF INAPPROPRIATE NUTRITION

Situations with inadequate nutrition, due to restricted or inappropriate diet or impaired nutrient absorption, are numerous. Despite their heterogeneous origins, they might share common metabolic consequences. Cow's milk allergy is the prototype of food allergy and may also be viewed as a model mechanism of any food intolerance and inappropriate nutrition in early infancy, when milk is the main nutrition.

Interestingly, we noticed a significant increase in urinary excretion of β-hydroxybutyrate in patients with CMA. β-hydroxybutyrate, along with acetoacetate, is a ketone body that is excreted to urine in larger amounts during ketosis, i.e. when body fats are liberated for energy metabolism. This occurs normally during fasting, prolonged physical exercise, or during high-fat (or low-carbohydrate) diet when the lipolysis of fats to acetoacetate exceeds the capacity of carbohydrate metabolism to produce oxaloacetate to be joined with acetoacetate in the citric acid cycle (Cahill 2006).

The infants in this study were not fasted. It is also reasonable to assume that the physical activity was equal in patients and controls, the mean age being 6 months. Thus, the rise in urinary ketones in patients later diagnosed with CMA may not be a sign of altered bacterial metabolism but, instead, reflect a metabolic change in the patients in response to prolonged inappropriate nutrition. The ketosis in these patients could be a chronic state similar to fasting, but due to inadequate use of

nutrition following intestinal inflammation resulting from intolerance to milk, the main nutrient in this age group.

It could also be argued that children less than one year of age with intestinal symptoms suggestive of food allergy would have had less varied diets with respect to the initiation of solid foods than their controls. Thus, food allergic children would be receiving more milk and, possibly, more fat – and their elevated urinary ketone bodies could only be due to a high-fat-low-carbohydrate diet. The study protocol assured that all infants were exposed to CM before the sample was taken, but their diet was not specified in other respects. Thus, this possible explanation, although unlikely, cannot be excluded.

9.6.2. FROM BACTERIAL METABOLITES TO METABOLIC PROFILING

CMA is a common condition but, unfortunately, its diagnosis is often challenging due to unspecific symptoms and laborious diagnostic protocols as explained earlier (5.2.). Recently, probiotics have been extensively studied (and marketed) in allergic diseases, but their role is still far from clear. In addition to their contribution to the composition of intestinal microbiota, probiotics also enhance intestinal barrier function, and some of their effects may be due to changes in the metabolic activity of intestinal microbiota following probiotic colonisation (Madsen et al. 2001, Rosenfeldt et al. 2004). Understanding the metabolic abnormalities and, eventually, the metabolic mechanisms of probiotic action in CMA could provide new approaches to the diagnosis and treatment of CMA.

9.6.2.1. From analysis of bacterial species to microbial metabolite recognition

Studying bacterial metabolites such as bacterial SCFA offers remarkable benefits in comparison to studying the bacteria themselves; namely, only 40% on bacterial species in human intestine are culturable outside the gastrointestinal tract (Sandin et al. 2009). Gene technology, e.g. 16S rRNA –based identification of different bacterial species now provides more sensitive means to study bacterial species themselves (Zoetendal et al. 2006); however, to understand bacterial function and host-microbe interactions, studying bacterial metabolites still remains indispensable.

Metabolites from the intestinal microbiota are not only relevant in gaining information about the microbial species. End products of bacterial metabolism also influence the host metabolism. Intestinal microbiota synthesise vitamin K for the host and participate to calcium and iron absorption. In addition, bacterial SCFA participate to host metabolism. Butyrate produced by intestinal bacteria has trophic properties to intestinal epithelium by serving as an energy source to colonocytes;

acetate and propionic acid modulate the host's glucose metabolism; all three acids stimulate proliferation and differentiation in intestinal epithelial cells (Guarner and Malagelada 2003, Guarner 2006).

9.6.2.2. Urinary versus faecal metabolite excretion; biologic variation related to age, diet and medication

According to previous studies, the development of allergy in children is associated with higher amounts of faecal SCFA, and a different SCFA profile with lower levels of i-butyrate, (i-)valerate, and propionate and more i-caproate in children later developing or already having allergy (Böttcher et al. 2000, Kalliomäki et al. 2001 a), Norin et al. 2004, Sandin et al. 2009). In our study, the urinary excretion of these acids was not changed in children with CMA; instead, we found higher urinary levels of β-hydroxybutyrate, adipate, isocitrate, homovanillate, suberate, tartarate, 3-indoleacetate and 5-hydroxyindoleacetate.

It is interesting that the urinary metabolic profile in our study does not seem to correlate with the previous faecal SCFA patterns in allergic infants. We compared food allergic children to atopic (eczematous) children without food allergies, as our aim was to find markers for inappropriate nutrition due to food intolerance; in previous studies, the focus has been on comparing allergic children to non-allergic ones. The fact that similar metabolic differences that we noticed have not been found in other studies with allergic children may strengthen the hypothesis that the differences we noticed were inherent to the intestinal food allergy itself, not just a by-product of atopic predisposition as different faecal SCFA patterns may be.

From studies on faecal SCFA patterns, it is known that factors such as age, diet and antibiotic or probiotic intake influence the results. According to Böttcher et al. (2000), age and the length of breast-feeding influence faecal SCFAs in under 9-month-old infants, but not in elder children. It is reasonable to assume that variation due to these factors could occur in urinary metabolite levels as well. In our study, antibiotic and probiotic intake were controlled. Except for the obligatory exposure to CM in some form, the diet of the patients and controls was not controlled in other ways, and some changes could arise from differences in the diet. However, as both the patients and controls were on average 6 months, their diets were fairly similar, mainly consisting of milk. The patients and controls in the study cohort as a whole were breast-fed for an equal time, and cow's milk was introduced at a similar age in all groups. Although unlikely, it cannot be excluded that the patients and controls of our study population, which was randomly selected form the larger study cohort, would have been different in these respects.

Naturally, the results of faecal and urinary metabolite profiles are not directly comparable: all of the metabolites measurable in faeces may not be excreted in

urine, or they may be metabolised by the host before urinary excretion. On the other hand, urinary metabolites may reflect a long-term metabolic state (Maher et al. 2007), whereas faecal SCFA profile may be more labile and subject changes following differences in the diet, received antibiotics, age etc (Böttcher et al. 2000).

We did not control for possible faecal contamination, which would influence urinary levels of bacterial SCFA. Faecal contamination may always take place when taking urine samples from infants using an adhesive urine bag, although the techniques we used (bag replacement every 2h, immediate cooling of the sample) minimise the risk. The technique of urinary sampling could not have been modified, as sterile techniques (catheter or suprapubic puncture) would not have been ethically justifiable in this patient group. However, it would be possible to involve urinary bacterial cultures in the study protocol to control for the possibility of faecal contamination of urine samples; still, it would leave the majority of bacterial species undetected, as approximately 40% of intestinal microbes are culturable (Sandin et al. 2009).

9.6.2.3. From urinary metabolites to metabolic profile and metabolomics

Urinary excretion of substances may provide an insight into a long-term metabolic balance. Urinary levels, however, represent the net sum of many different metabolic processes over a longer time course. Rather than making statements about levels of single substances in urine, it seems necessary to look after urinary patterns or urinary profiles, which is increasingly being done in metabolomics (Theodoridis et al. 2012). The idea is to scan by a sensitive method – either liquid or gas chromatography/ mass spectrometry -based methods or nuclear magnetic resonance spectroscopy – for all possible metabolites in a biologic sample and then look after specific profiles or patterns by multivariate statistical analysis.

Analysing metabolic profiles rather than single metabolites makes it possible to take a holistic approach to metabolism and see it as a sum of multiple parallel chemical reactions, which it truly is. This field in metabolic studies is still new, and studies from different laboratories not yet directly comparable (Theodoridis et al. 2012). Our small study population did not make it possible to make relevant statistical analysis of the true urinary metabolic profiles, not to mention the metabolome, of our patients, but this could be feasible in further studies.

9.6.3. POSSIBILITIES FOR DIAGNOSIS

Food allergies cause chronic inflammation of the gut (Mansueto et al. 2006), which affects intestinal absorptive function and increases intestinal permeability by disrupting the epithelial barrier (Macdonald and Monteleone 2005). Atopy and

allergies are also associated with changes in intestinal microbiota (Bjorksten et al. 1999, Kirjavainen et al. 2001) and its metabolic function (Ouwehand 2007). The causality between food allergies, changes in intestinal microbiota and increased intestinal permeability are not clear; it seems that altered microbiota induces intestinal inflammation leading to altered intestinal permeability, but individual immunologic factors then determine whether changes in permeability result in the development of tolerance or intolerance. Ideally, the absorption – or leakage –of normally unabsorbed compounds from the gut could be used as a marker of altered intestinal permeability and bacterial metabolism, and even as a diagnostic tool for food allergies.

9.6.3.1. Urinary lactulose/mannitol ratio as a marker of intestinal permeability and food allergy

Children with food allergies have different intestinal permeability to specific compounds as compared to healthy controls (Jackson et al. 1981); the permeability to small molecules (e.g. mannitol) seems to be identical, but the permeability to large, normally unabsorbed, molecules (e.g lactulose) increased (Andre et al. 1987, Laudat et al. 1994). The ratio of permeability to lactulose and mannitol (measured as the recovery of lactulose and mannitol in urine after oral lactulose and mannitol load) has been suggested to be a marker of allergy-related change in intestinal permeability and, eventually, as a diagnostic method (Andre et al. 1987, Dupont et al. 1989, Laudat et al. 1994).

Many studies, however, show opposing results and no change in lactulose/mannitol ratio in food allergy (Kuitunen et al. 1994, Catassi et al. 1995). The conflicting results may be partly explained by the large biologic individual variation in lactulose/mannitol ratios. In addition, all infants have increased intestinal permeability, which then gradually decreases with age. This causes difficulties in the interpretation of lactulose/mannitol ratios in young children. Thus, it has not been possible to apply the findings of the lactulose-mannitol test for food allergy diagnosis in a reliable way suiting clinical practise.

9.6.3.2. Increased urinary organic acid excretion in CMA: leaking intestine or altered microbiota?

Consistent with the theory of the increased intestinal permeability in food allergies (Dupont et al. 1989), all urinary organic acids that differed significantly between eczema patients with and without CMA in our study were higher in CMA patients. Nevertheless, despite the changes in urinary organic acid excretion, infants with

CMA in our study did not have other evidence of increased intestinal permeability, as their lactulose/mannitol ratio was similar to controls (Kuitunen et al. 2007). This suggests that the changes we noticed in urinary metabolic end products would be due to differences in intestinal bacterial metabolism rather than a general increase in intestinal permeability.

On the contrary, the effects of LGG treatment to urinary organic acids in CMA patients are suggestive of intestinal permeability playing a role in urinary organic acid excretion. Even if statistical significance was not reached in this small study population, the excretion of urinary organic acids decreased towards the control level after probiotic treatment. This supports the idea of the increased intestinal permeability related to food allergies being at least partly reversible. One could state that LGG treatment normalised intestinal flora and its metabolism. These results are, however, in line with the results of the lactulose-mannitol test, where LGG treatment reduced the permeability to mannitol in infants with CMA, and a similar trend was observed for the permeability to lactulose (Kuitunen et al. 2007). Although not studied, it seems unlikely that changes in the composition or metabolism of intestinal flora would affect urinary lactulose and mannitol levels; instead, a change in intestinal permeability – due to low-grade inflammation induced by LGG, as Kuitunen et al. (2007) speculate – would be a more probable explanation.

The increase in urinary tartarate excretion that we noticed in patients with CMA is particularly interesting, as tartarate is not produced in human metabolism (Shaw et al. 1995). If confirmed in further studies, urinary tartarate could possibly serve as a marker for increased intestinal permeability and/or changes in microbial metabolism. Yeasts may produce tartarate (Shaw et al. 1995) and one could speculate that an explanatory factor for the elevated tartarate excretion in children with CMA would be intestinal yeast overgrowth due to changes in intestinal microbiota. It seems, however, that dietary intake still plays the most important part (Lord et al. 2005) in human tartarate intake (and, possibly, excretion).

If confirmed and specified with further research, the different patterns of urinary organic acid excretion between allergic and non-allergic infants could provide an innovative new approach to the diagnosis of CMA in infants.

9.6.3.3. Could urinary metabolic profiling distinguish between different allergies?

If food allergies cause measurable metabolic and permeability changes in the intestine, these would probably not be allergen-specific; instead, the metabolic differences would be general markers of food intolerance leading to inflammation and altered microbial balance in the intestine. In our study with on average 6-month-

old infants this was fairly well controlled, as in this age group, milk is by far the most important nutrient and if food intolerance in this age group is present, CMA is probable. As the differences in urinary metabolic profiles seemed to be reversible with LGG treatment, it would be interesting to study whether similar changes towards the control level would be seen after allergen (CM) elimination. An elimination – exposure –protocol with repeated urinary metabolic profile analyses could, then, help to distinguish between different allergens in subjects with a more varied diet.

10. CONCLUSIONS

This thesis has focused on biochemical changes in both inherited and acquired situations with significant metabolic disturbances. These changes are seen as metabolic markers of the underlying pathogenetic processes, offering new insights into disease mechanisms and, with further research, even therapeutic potential.

The main conclusions of the study are the following:

(1) Thiol metabolism is altered in children with several inherited metabolic diseases associated with defects in energy metabolism and nutrition. The changes in thiol metabolism in these metabolic diseases may partly be due to nutritional factors, but it seems that more complex disease mechanisms are involved.

(2) The alterations in plasma thiols and their redox state in organic acidaemias, mitochondrial diseases, hypoglycaemia and homocystinuria are suggestive of oxidative stress, which has previously been shown to play a role in the pathogenesis of complications of these diseases. As a new finding, the results of this series of studies show that oxidative stress may be present already at a stable phase of these metabolic diseases and thus truly contribute to the disease processes.

(3) CMA is associated with measurable changes in urinary levels of end products of metabolism. These changes seem to reflect increased intestinal permeability and, possibly, differences in intestinal bacterial metabolism. The changes in urinary metabolic profile in CMA may also be seen as metabolic markers of inappropriate nutrition in early infancy.

11. ACKNOWLEDGEMENTS

This study was carried out at the Children's Hospital, University of Helsinki. I wish to express my gratitude to Professors Helena Pihko, Mikael Knip and Martti Siimes, the current and former Heads of the department. Furthermore, I would like to thank Docent Jari Petäjä and Dr. Veli Ylitalo, the current and past Heads of the Department of Gynaecology and Pediatrics of the Helsinki University Central Hospital, and Docent Eero Jokinen, Head of the Department of Pediatrics, for giving me access to the research facilities. Markku Heikinheimo, Professor of paediatrics, Head of the Department of Clinical Medicine and former Head of the National Graduate School of Clinical Investigation, and Docent Jussi Merenmies, present Head of the Pediatric Graduate School, have created a supportive environment to learn research at the Pediatric Graduate School in the University of Helsinki. I am very thankful for their help and enthusiasm.

I am deeply thankful to my supervisor, Docent Risto Lapatto, who guided and encouraged me tirelessly throughout this ten-year long project and always had time and patience for my questions. I cannot imagine a more pedagogic supervisor; his view of the thesis as a process where learning and education play the most important role could be extended to many other projects in life.

I wish to thank Docent Päivi Keskinen and Docent Matti Nuutinen for the true interest they showed and for the constructive comments and valuable suggestions they gave when reviewing the thesis. I likewise warmly thank Professor Markku Heikinheimo and Docent Päivi Miettinen, the follow-up group for my thesis, for advice and encouragement during several years.

I have greatly appreciated the help of Professor James Leonard, Dr. Khalid Hussain and Dr. Shamima Rahman from the Great Ormond Street Hospital, London, UK, and Docent Mikael Kuitunen and Dr. Mirva Viljanen from the Skin and Allergy Hospital at Helsinki University Central Hospital, when writing the manuscripts of the original publications. The opportunity to work with and learn from such experts has been a privilege.

I am thankful to my colleagues in the Children's Hospital and my former colleagues in the South Carelian Central Hospital for support. Moreover, thank you for your endless faith in my skills – none of you has ever asked why I did not choose an easier subject for my thesis! I specially wish to thank the now-colleagues Kirsi Ylä-Tuuhonen and Tytti Usmi for their contribution to the laboratory analyses and Ms. Sari Lindén and Ms. Ritva Löfman for their excellent technical assistance. I am also very grateful to Pietu Pohjalainen for his indispensable and immediate help when drawing some figures for the publications.

I am grateful to my father-in-law, Docent Tapani Salmi, for telling me about the existence of the mythical π time factor in research projects. Without this and his inspiring example as a 150 % scientist and 175 % clinician, I would have given up all hope about ever finishing. Sill, my interest to science is due to my father Heikki Miettinen's unforgettable performance about the function of the Solar System some 25 years ago. Thank you for showing me that complicated things can be understood – and if not quite understood, they still turn into a good spectacle.

Almost last but certainly not the least, I want to thank all my friends and relatives; I have always felt lucky to be surrounded by so many well-wishing and brilliant people. Three persons, however, deserve even more praise: my mother Pirkko Miettinen, for her love, help and optimism, and for still knowing what I think before I do; my husband Lauri, for everything, but especially for providing me with what its known in Finnish as feet on the earth –attitude; and my smiling daughter Suvi, for being Suvi.

The Finnish Medical Foundation and South Carelian Medical Association financially supported this study.

Thank you!

Helsinki, April 2012

Heli Salmi

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